AD_____

Award Number: DAMD17-98-1-8015

TITLE: PAI-1 Gene as a Target for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Paul J. Higgins, Ph.D.

CONTRACTING ORGANIZATION: Albany Medical College
Albany, New York 12208

REPORT DATE: April 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2002	Final (1 Apr 98 - 31 Mar 02)

4. TITLE AND SUBTITLE
PAI-1 Gene as a Target for Breast Cancer Therapy

5. FUNDING NUMBERS
DAMD17-98-1-8015

6. AUTHOR(S)
Paul J. Higgins, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Albany Medical College
Albany, New York 12208
E-Mail: higginp@mail.amc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

This final report details in vitro and in vivo growth characteristics of stable lines of human breast carcinoma cells (developed throughout this study) that synthesized varying levels of plasminogen activator inhibitor type-1 (PAI-1) as a result of transfection with expression vectors bearing PAI-1 cDNA inserts cloned in sense and antisense orientations. A selected set of tumor cell lines (based on level of PAI-1 expression perturbation) was completely characterized with regard to specific in vitro motile traits (chemokinesis, chemotaxis, 2-D planar migration, barrier invasion). PAI-1 synthesis was required for tumor cell migration in directed assays since antisense targeting effectively suppressed cell motility. These results were confirmed with neutralizing PAI-1 antibodies and exogenously-supplied recombinant PAI-1 protein. A expression vector was created in which PAI-1 promoter sequences were used to drive expression of a chimeric PAI-1-GFP insert. This vector confirmed PAI-1 expression in locomoting cells and PAI-1 deposition into migration trails suggesting its use as a tool to track metastatic tumor cells in vivo. PAI-1 down-regulation slowed human breast cancer implant growth in nude mice and inhibited lung colonization suggesting a therapeutic benefit to PAI-1 gene targeting.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Gene therapy, breast cancer, PAI-1, growth control

15. NUMBER OF PAGES
67

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Cancer invasion and metastasis are complex processes in which controlled and focalized extracellular matrix (ECM) degradation facilitates cellular migratory activity. ECM turnover is regulated temporally and spatially by the concerted action of several interacting proteolytic systems including, most prominently, the generation of plasmin by the urokinase (uPA)-dependent pathway of plasminogen activation (Dano et al., 1994; Andreasen et al., 1997). Plasmin, in turn, degrades the ECM directly as well as indirectly by activating latent metallo-proteinases (e.g., MMP1, 2, 9) (Dano et al., 1987; Vaheri et al., 1990; Pollanen et al., 1991). Within this context, plasminogen activator inhibitor type-1 (PAI-1) plays a primary role as a negative regulator of the plasmin-based pericellular proteolytic cascade by complexing to, and inhibiting the catalytic activity of, free as well as receptor-bound uPA (Blasi et al., 1987; Andreasen et al., 1997). This cascade directly influences the overall tissue site proteolytic balance and is a critical determinant in directed cell movement, extracellular matrix (ECM) invasion and tumor metastasis (Pepper et al., 1987; 1992; Okedon et al., 1992; Jeffers et al., 1996; Mazzier et al., 1997; Wysocki et al., 1999; Providence et al., 2000, 2002; Reijerkerk et al., 2000; Brooks et al., 2001). Recent studies in PAI-$1^{-/-}$ mice confirmed the importance of PAI-1 in tumor spread and the associated angiogenic response (Bajou et al., 1998, 2001; Gutierrez et al., 2000; McMahon et al., 2001; Stefansson et al., 2001). A critical balance between proteases and their specific inhibitors, thus, has been suggested as necessary to maintain an ECM scaffold structure compatible with efficient cellular locomotion (Bajou et al., 2001). Cell type-specific synthesis and subcellular targeting of PAI-1 and uPA, moreover, are important considerations in modulation of the pericellular proteolytic environment. Temporal changes in the expression, focalization, and/or relative activity of this protease/inhibitor pair influence cell migration either as a direct consequence of ECM barrier proteolysis or by modulating cellular adhesive interactions with the ECM (Ciambrone and McKeown-Longo, 1990; Blasi, 1996, 1997; Bajou et al., 2001). This has direct relevance to outcomes in human breast cancer. Clinical studies have demonstrated that, in general, elevated tumor levels of uPA, its cell surface receptor (uPAR), and PAI-1 are conducive to breast tumor metastasis and associated with poor disease prognosis (Schmidt et al., 1992; Duffy, 1996; Pedersen et al., 1994). The role of PAI-1 as a determinant in aggressive growth behavior is particularly important in mammary carcinoma (Costantini et al., 1996; Gandolfo et al., 1996; Mayerhofer et al., 1996; Fersis et al., 1996; Torre and Fulco, 1996; Foekens et al., 1995; Cufer et al., 2002; Look et al., 2002; Fox et al., 2001). More recently, PAI-1 has been shown to be markedly promigratory for invasive breast cancer cells, an effect attenuated by direct inhibition of PAI-1 function (Chazaud et al., 2002). These data highlight the potential relevance of PAI-1 modulation as a means to treat human breast cancer and complement our own work on genetic targeting of PAI-1 mRNA transcripts with subsequent attenuation of cellular motile traits (Providence et al., 2000). The purpose of this work, therefore, is to utilize a molecular approach to manipulate levels of PAI-1 expression in human breast carcinoma cells for the purpose of: (1) generating panels of genetically-engineered human breast cancer cells which vary in levels of PAI-1 expression/synthesis, (2) assessing the *in vitro* growth characteristics of these cells (specifically as regards invasive behavior), and (3) evaluating the usefulness of the PAI-1 gene as an *in vivo*

therapeutic target. To do this, we have constructed vectors bearing full-length PAI-1 cDNA inserts. Expression of this cDNA, cloned in both sense and antisense orientations, is under control of various, tandemly-linked, copies of (MYC-responsive) E-box promoter sequences to take advantage of the endogenous breast cancer MYC protein as a means to regulate transcriptional strength. Such utilization of tumor-associated anomalies in transcription factor (i.e., MYC) expression to direct genetic-based intervention therapies is a novel approach to cancer treatment. This strategy, moreover, addresses the more aggressive breast tumor cell type, the highly proteolytically active potentially metastatic cells, for specific therapy. Our approach takes advantage of the amplified MYC expression typical of breast carcinoma to transactivate transfected vectors resulting in high level sense and antisense PAI-1 transcript production. These studies will provide information critical to the eventual design of tumor type-appropriate targetable delivery systems for genetic therapy of breast cancer.

Body of Report

We had originally hypothesized that over-expression of PAI-1 by malignant epithelial cells was an important aspect in the progression of a relatively indolent localized tumor to an invasive, highly metastatic, phenotype. Based on data summarized in the **Introduction,** our working model is that temporal changes in the expression, focalization, and or relative activity of urokinase PA/PAI-1 may influence cellular migration and invasive traits either as a direct consequence of ECM barrier proteolysis or by modulating cellular adhesive interactions (via cycles of adhesion/de-adhesion) with the supporting matrix. This is consistent with recent findings indicating that PAI-1, specific integrins, and urokinase PA function coordinately to regulate adhesive events important in the control of cell movement (Ossowski and Aguirro-Ghiso, 2000). Our recently published observations (Providence et al., 2000; Kutz et al., 2001) provide initial experimental verification of this concept using a relatively simple culture system of induced epithelial cell locomotion over a planar substrate. In this model, PAI-1 expression "targeting" with antisense vectors attenuated both PAI-1 protein synthesis and 2-D planar migration. Two aspects of this work are directly relevant to the working hypothesis of the present grant. Epithelial cells exhibit a location-specific distribution of PAI-1 expressing cells (i.e., only locomoting cells express this SERPIN) and down-regulation of PAI-1 synthesis and its matrix deposition in epithelial cells transfected with a PAI-1 antisense expression vector significantly impaired cell migration. Most importantly, it was possible to "rescue" a PAI-1 functionally-null, motile-deficient, epithelial cell line by transfection with a PAI-1 expression vector (Providence et al., 2000). Rescued cells expressed approximately normal levels of PAI-1 and efficiently locomoted in response to a denuding stimulus. Moreover, we most recently have constructed two PAI-1-GFP expression vectors regulated by an upstream CMV promoter or 800 bp of endogenous PAI-1 promoter sequences (**Figure 1**). This vector was used to confirm deposition of PAI-1 into cellular migration tracks. We have taken advantage of the availability of this vector to engineer additional breast cancer lines that express PAI-1-GFP (described below as well as in **Key Research Accomplishments**). This provides a marker to "track" highly aggressive human breast cancer cells *in vitro* or following

transplantation into nude mice thus providing a sensitive tag to detect micrometastases. Gene expression patterns, adhesion and motility of transfectant MCF-7 and MDA-MB-231 cells were assessed for each (CMV-, PAI-1- or E-box-driven) construct.

The **Specific Aims** of this proposal were (1) to generate transfectants of human breast carcinoma cells that vary in PAI-1 expression at both the mRNA and protein levels and to characterize the *in vitro* growth traits of these genetically-engineered cells, (2) to assess the *in vivo* growth characteristics of human breast carcinoma cells designed to express different levels of PAI-1 using the results obtained in **Aim 1** to identify the most important candidate clones to evaluate, and (3) to initiate studies to assess the targetability of *in vivo*-implanted human breast tumor cells with potential therapeutically-relevant vectors as concluded from results obtained on work directed to **Aims 1 and 2.**

To achieve these aims, the goals in **Tasks 1 and 2** in the originally proposed **Statement of Work** were as follows:

Task 1: to assess the effects of vector-directed PAI-1 expression on *in vitro* growth traits of human breast carcinoma cells.

(a) Develop a panel of transfectant MCF-7 (estrogen responsive) and MDA-MB-231 (estrogen receptor negative) breast carcinoma cells which express differing levels of vector-driven PAI-1 mRNA and protein.
(b) Perform assays to assess the in vitro growth characteristics of the individual transfectant cell lines.
(c) Correlate levels of PAI-1 gene expression for each transfectant cell line with specific *in vitro* growth traits.

Task 2: to assess the effects of vector-driven PAI-1 expression on *in vivo* traits of the individual transfectant human breast carcinoma cell lines.

(a) Perform assays to determine the *in vivo* growth traits and metastatic characteristics of the individual transfectant cell lines.
(b) Confirm maintenance of vector sequences and level of PAI-1 gene expression in any resultant tumors.
(c) Initiate a feasibility study to evaluate vector "targetability" of *in vivo* breast carcinoma implants.

We have completed work designed to address **Task 1.** A total of 32 different stable transfectant breast carcinoma cell lines were developed; 17 MCF-7-derived lines bearing a positive sense PAI-1 expression vector (either Rc/CMVPAI or Rc/E-Box PAI) and in which PAI-1 mRNA levels varied from 0.8 ± 0.1 to 49.6 ± 9.0 fold over MCF-7 parental or vector without insert controls and 15 MDA-MB-231 antisense PAI-1 (either Rc/CMVIAP or Rc/E-BoxIAP) vector-derived lines in which PAI-1 mRNA abundance was down-regulated by 11 to 92% compared to exponentially growing parental controls or cells transfected with vector without insert. A panel of 6 transfectant lines (for both the MCF-7 and MDA-MB-231 derived cell types) was selected for analysis of in vitro growth traits based on levels of PAI-1 expression significantly different from either of the parental strains. A quantitative analysis of these growth traits is summarized in **Table 1.**

These data support the concept that PAI-1 is an important promigratory effector in breast tumor cells consistent with observations in other epithelial cell systems (e.g., Providence et al., 2000; see also **Figure 2**). Additional phenotypic characteristics of these cell lines is provided in **Key Research Accomplishments**. A most important development during this last year was our ability to construct several chimeric vectors in which a PAI-1-GFP fusion insert was cloned under control of either CMV promoter sequences (for constitutive insert expression) or PAI-1 promoter sequences (for inducible expression) (the PAI-1 promoter-driven PAI-1-GFP insert construct is detailed in **Figure 1**). We have successfully created epithelial transfectants that express, process and transport PAI-1-GFP in manner identical to the endogenous PAI-1 protein. These genetically-"tagged" cells can be tracked *in vivo* for unambiguous evaluation of the role of PAI-1 in tumor cell invasion *in vivo* as well as in the creation of micrometastatic deposits. A new proposal is currently in preparation (for submission to the USAMRMC as an IDEA grant) that details use of these new vectors to trace *in vivo* metastases of human breast carcinoma implants. Transient transfection approaches (employing the PAI-1 promoter-PAI-1-GFP expression construct; **Figure 1**) and use of neutralizing PAI-1 antibodies confirmed the importance of PAI-1 in breast carcinoma motility. Direct PAI-1 activity blockade with neutralizing antibodies served to evaluate the potential of PAI-1 function targeting as an alternative approach to modulate breast tumor cell invasive traits. PAI-1 neutralizing antibodies promoted substrate detachment (or blocked cell-to-substrate adhesion/spreading) and inhibited cell locomotion (in both 2-D "scrape-injury" planar models and 3-D Matrigel invasion assays) **(Table 2)**. In contrast, breast tumor cell substrate attachment and motility was significantly stimulated by addition of exogenous active PAI-1 protein. Vector-driven PAI-1 synthesis in MCF-7 and MDA-MB-231 cells increased migratory rate in both the 2-D and 3-D systems whereas targeted down-regulation of PAI-1 synthesis in MDA-MB-231 transfectants markedly attenuated both cell-to-substrate adhesion and migratory activity, consistent with results using neutralizing antibodies. Creation of high level PAI-1 over-expressing MDA-MB-231 cells with the inducible PAI-1-GFP construct resulted in extensive accumulation of GFP in both membrane ruffles and the cellular undersurface (similar to that evident in transfected RK epithelial cells; i.e., **Figure 1**) and a significant increase (over parental MDA-MB-231 cells) in 2-D planar locomotion and 3-D invasive traits **(Table 3)**.

We have also largely completed work designed to address Task 2. The 7 MDA-MB-231-derived cell lines characterized in detail in **Table 1** were implanted subcutaneously (3×10^6 cells/injection site) in the region of the mammary fat pad into NCR nu/nu mice. The incidence of tumor formation over a specific time period (10 weeks) and the ability to metastasize (to the adominal cavity, lungs or liver) were determined at necropsy. Tumor volume (in mm^3) was estimated by caliper measurements of the resected primary mass. The MDA-MB-231-derived panel of tumor cells **(Table 1)** was specifically selected for this analysis since (1) metastasis due to implantation of this cell line is associated with adaptation to high uPA expression (Seddighzadeh et al., 1999), thus, making assessments of the primary inhibitor of uPA activity (i.e., PAI-1) directly relevant to tumor behavioral outcomes and (2) levels of PAI-1 expressionas a consequence of constitutive antisense transcript expression differed by over an order of magnitude **(Table 1)**. Decreased PAI-1 synthesis (confirmed most recently using

Western blotting and PAI-1-specific rabbit antibodies) by MDA-MB-231 transfectants was most effectively obtained with the PAI-1 antisense constructs in which expression was driven by E box-containing upstream promoter elements (e.g., EBIAP clones 3 and 21). Those clones exhibiting the most significant PAI-1 down-regulation produced the slowest growing tumors upon subcutaneous transplantation into nude mice. Unlike parental MDA-MB-231 cells or transfectants in which PAI-1 levels were not appreciably decreased (e.g., clone CMVIAP-6) and which exhibited the capacity to form lung nodules, clones EBIAP-3 and 21 were non-metastatic (summarized in **Table 4**). We had originally intended to directly confirm levels of PAI-1 mRNA transcript expression by PCR (as a measure of *in vivo* efficacy of antisense vector function) in tumors that resulted from transplantation of genetically-engineered MDA-MB-231 transfectants. This was determined not to be possible for two reasons: (1) the angiogenic response to transplanted tumors, regardless of size, brought PAI-1^{+} endothelial cells into close proximity of the tumor mass making it impossible to distinguish MDA-MB-231- from (endothelial) host-derived PAI-1 mRNA by this technique and (2) the high incidence of necrotic "cores" evident upon growth of the EBIAP clones 2 and 21 in nude mice made recovery of PCR quality RNA difficult. To avoid problems associated with issue (1) above, we are planning to explore the use of the PAI-$1^{-/-}$ mouse as host for future transplantation studies with this panel of MDA-MB-231 transfectants. We have already established a breeding colony of PAI-$1^{-/-}$ at this institution for this purpose. To address issues (1 and 2) above, future proposals will suggest use of in situ hybridization with species-specific PAI-1 probes to distinguish human tumor cell from mouse host endothelial PAI-1 transcripts (in the event that PAI-$1^{-/-}$ mice prove unsuitable hosts for human breast tumor cell xenografts) combined with harvest of relatively early stage, minimally-necrotic, tumor tissue.

Key Research Accomplishments

A panel of transfectant human breast carcinoma cell lines (derived from both MCF-7 and MDA-MB-231 parental stocks) was developed that varied in vector-driven synthesis of PAI-1 mRNA and protein. These cell lines were suitable for conduct of all in vitro and in vivo growth assessments as originally proposed.

Comparisons between in vitro growth traits and PAI-1 expression as a function of cell growth activation indicated that MCF-7 cells were low to non-PAI-1 expressing regardless of proliferative stages (i.e., quiescent vs. cycling G1 vs. exponentially growing) whereas MDA-MB-231 cells expressed relatively high levels of PAI-1 mRNA/protein, particularly in serum-supplemented culture conditions. This differential in PAI-1 synthesis correlated with the low intrinsic motility (i.e., directed migration across denuded planar surfaces) of MCF-7 cells compared to the highly migratory phenotype of MDA-MB-231 carcinoma cells. A manuscript is currently in preparation detailing this expression defect in the MCF-7 lineage and reprints will be forwarded to the USAMRMC when received. A related series of studies indicated that the relatively high endogenous level of PAI-1 synthesized by human breast tumor cells of the MDA-MB-231 lineage could be inhibited by the pharmacologic compound PD98058 suggesting

that mitogen-activated kinases were important transducers of signals that regulated expression of this motogenic protein.

Extent of cell spreading on vitronectin-coated bacteriological culture dishes was approximately inversely related to the level of PAI-1 expressed by transfected MCF-7 cells (i.e., MCF-7 cells attached and spread onto vitronectin; the extent of cell spreading decreased with increasing vector-driven expression of PAI-1). There was no direct quantitative relationship between MCF-7 cell spreading on fibronectin and PAI-1 levels although high PAI-1 expressing MCF-7 cells were flatter on fibronectin-coated dishes compared to their low-PAI-1 expressing counterparts. MDA-MB-231 parental cells that constitutively synthesized abundant PAI-1 mRNA/protein were highly motile and formed extensive membrane ruffles (indicative of a locomoting phenotype). PAI-1 antisense vector-directed down-regulation of PAI-1 synthesis in clonal isolates of MDA-MB-231 cells resulted in modified cell spreading (compared to parental controls), loss of membrane ruffling and decreased migration across a planar substrate.

Increased PAI-1 expression in PAI-1 sense vector MCF-7 transfectants resulted in an increase in random migration as well as augmenting both the chemokinetic and chemotactic index. PAI-1 expression was necessary for elaboration of the migratory/invasive phenotype in MDA-MB-231 cells as a vector-driven down-regulation of PAI-1 synthesis in this cell type reduced the fraction of cells capable of random, chemokinetic and chemotactic migration. These data are summarized in **Table 1**.

Exogenously-supplied recombinant PAI-1 protein (either wild-type or the long-lived stabilized PAI-1 P14 mutant) significantly enhanced substrate attachment, 2-D planar migration and barrier invasion traits of MDA-MB-231 breast tumor cell. Consistent with these findings and previously published results, use of PAI-1 neutralizing antibodies effectively inhibited cellular adhesion (i.e., attachment and spreading) as well as migration in both 2-D and 3-D assay systems.

Breast tumor cells were transfected with expression constructs in which synthesis of a chimeric PAI-1-GFP insert driven by either a CMV promoter or PAI-1 promoter sequences. These cell express, process, and secrete a PAI-1-GFP fusion protein that has biological characteristics identical to that of the endogenous PAI protein with synthesis patterns identical to those identified in transfected RK cells (**Figure 1**). These cells will be important in evaluation of micrometastases.

Targeted down-regulation of PAI-1 synthesis in MDA-MB-231 human breast carcinoma cells with the PAI-1 EB-driven antisense construct inhibited tumor growth and prevented the development of lung nodules.

Reportable Outcomes

1. Manuscripts, abstracts, and presentations that directly resulted from support of this project by the Department of the Army under grant DMD17-98-1-8015 are listed in the Appendix under "Additional Requested Information".

2. Development of cell lines:

All MCF-7 and MDA-MB-231 cell lines and their transfectant derivatives will be maintained in Dr. Higgins' laboratory. These cells will be made available upon request to members of the scientific community engaged in breast cancer research.

3. The development of several potentially useful vectors and chimeric constructs was possible upon funding of this program. Maps of these vectors and details with regard to use in breast cancer and other cell types will be provided upon request.

Conclusions

The following is a summary of the conclusions of the present report and the implications of the results obtained.

PAI-1 expression levels in indolent breast carcinoma cells (e.g., MCF-7) are low relative to the abundant expression of PAI-1 mRNA/protein characteristic of highly aggressive and metastatic breast tumor cell types (e.g., the MDA-MB 231 cell line) suggesting that expression is linked to the biological behavior of a particular breast tumor rather than simply to cellular proliferation. These results suggest that assessments of PAI-1 synthesis in breast cancers may represent an important parameter for patient classification. Indeed, a survey of the most recent studies that address this issue (summarized in the **Introduction**) has clearly indicated that tumor PAI-1 levels are not only important in patient stratification but that such levels are predictive of relapse, extent of disease-free survival, and indicative of the tumor angiogenic response. Patients presenting with high PAI-1-positive primary breast tumors may benefit from more aggressive therapy and post-operative surveillance. Analysis of our transfectant tumor cell lines additionally indicates that high levels of PAI-1 expression clearly modulates cell-to-matrix protein attachment properties and facilitates the elaboration of an invasive phenotype. Our studies detailing the effect of molecular targeting of PAI-1 gene expression in epithelial cells on induced directional migration into scrap-denuded "wounds" (Providence et al., 2000) is consistent with the present findings implicating PAI-1 as an essential element in the migratory apparatus of breast carcinoma cells. Our new technologic advance that enables us to actually visualize a PAI-1-GFP fusion protein in the migration "trails" of transfected cells during the real time of cell movement reinforces our gene targeting findings. The ability to influence both the rate of tumor growth and the metastatic capacity of human breast carcinoma cells by PAI-1 expression targeting, moreover, highlights the importance of this protein in carcinoma progression and

provides new insights into the previously paradoxical relationship between high tumor levels of PAI-1 and poor prognosis. Collectively, these data support the original hypothesis that the PAI-1 gene represents a reasonable (and now clearly an accessible) therapeutic target in the treatment and management of aggressive human breast cancers. Our work has also afforded an important opportunity to create genetically-engineered human breast cancer lines and targeted vector systems that we believe will be critical tools for future studies on gene therapy approaches to breast cancer management.

References

Andreasen PA, Kjoller L, Christensen L, Duffy MJ (1997) The urokinase-type plasminogen activator system in cancer metastasis; a review. Int J Cancer 72; 1-22.

Bajou K, oel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D, Foidart JM (1998) Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat med 4, 923-928.

Bajou K, Masson V, Gerard RD, Schmitt PM, Albert V, Praus M, Lund LR, Frandsen TL, Brunner N, dano K, Fusenig NE, Weidle U, Carmeliet G, Loskutoff D, Collen D, Carmeliet P, Foidart JM, Noel A (2001) The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications of antiangiogenic strategies. J Cell Biol 152, 777-784.

Blasi F, Vassalli JD, Dano K (1987) Urokinase-type plasminogen activator; proenzyme, receptor, and inhibitors. J Cell Biol 104; 801-804.

Blasi F (1996) The urokinase receptor and cell migration. Sem Thromb Hemost 22; 513-516.

Blasi F (1997) uPA, uPAR, PAI-1: key intersection of proteolytic, adhesive and chemotactic highways? Immunol Today 18; 415-417.

Brooks TD, Slomp J, Quax PHA, De Bart ACW, Spencer MT, Verheijen JH, Charlton PA (2001) Antibodies to PAI-1 alter the invasive and migratory properties of human tumour cells in vitro. Clin Exp Metastasis 18, 445-453.

Chazaud B, Ricoux R, Christov C, Plonquet A, Gherardi RK, Barlovatz-Meimon G (2002) Promigratory effect of plasminogen activator inhibitor-1 on invasive breast cancer cell populations. Am J Path 160, 237-246.

Ciambrone GJ, McKeown-Longo PJ (1990) Plasminogen activator inhibitor type 1 stabilizes vitronectin-dependent adhesions in HT-1080 cells. J Cell Biol 111; 2183-2195.

Costantini V, Sidoni A, Deveglia R, Cazzato OA, Bellezza G, Ferri I, Bucciarelli E, Nenci GG (1996) Combined overexpression of urokinase, urokinase receptor, and plasminogen activator inhibitor-1 is associated with breast cancer progression. Cancer 77;1079-1088.

Cufer T, Vrhovec I, Borstnar S (2002) Prognostic significance of plasminogen activator inhibitor-1 in breast cancer, with special emphasis on locoregioal recurrance-free survival. Int J Biol Markers 17, 33-41.

Dano K, Behrendt N, Brunner N, Ellis V, Plough M, Pyke C (1994) The urokinase receptor. Protein structure and role in plasminogen activator and cancer invasion. Fibrinolysis 8: 189-203.

Duffy MJ (1996) Proteases as prognostic markers. Clin Cancer Res 2; 613-618.

Fersis M, Kaufmann M, Karmer MD, Wittmann G, Wallweiner D, Basert G, (1996) Prognostic significance of plasminogen activator and its inhibitor PAI-1; predictors of poor response to tamoxifin therapy in recurrent breast carcinoma. J Natl Cancer Inst 87; 751-756.

Foekens JA, Look MP, Peters HA, van Putten WL, Portengen H, Klijn JG (1995) Urokinase-type plasminogen activator and its inhibitor PAI-1: predictors of poor response to tamoxifen therapy in recurrent breast cancer. J Natl Cancer Inst. 87, 751-756.

Fox SB, Taylor M, Grondahl-Hansen J, Kakolyris S, Gatter KC, Harris AL (2001) Plasminogen activator inhibitor-1 as a measure of vascular remodeling in breast cancer. J Path 195, 236-243.

Gandolfo GM, Conti L, Vercillo M (1996) Fibrinolytic components as prognostic markers in breast cancer and colorectal carcinoma. Anticancer Res 16;2155-2159.

Gutierrez LS, Schulman A, Brito-Robinson T, Noria F, Ploplis VA, Castellino FJ (2000) Tumor development is retarded in mice lacking the gene for urokinase-type plasminogen activator or its inhibitor, plasminogen activator inhibitor-1. Cancer Res 60, 5839-5847.

Jeffers M, Rong S, Vande Woude GF (1996) Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signaling in human cells concomitant with induction of the urokinase proteolysis network. Mol Cell Biol 16, 1115-1125.

Kutz SM, Providence, KM, Higgins, PJ (2001) Antisense targeting of c-*fos* transcripts inhibits serum-and TGF-β1-stimulated PAI-1gene expression and directed motility in renal epithelial cells. Cell Motil Cytoskeleton 48,163-174.

Look MP et al (2002) Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients. JNCI 94, 116-128.

McMahon GA, Petitclerc E, Stefansson S, Smith E, Wong MK, Westrick RJ, Ginsburg D, Brooks PC, Lawrence DA (2001) Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. J Biol Chem 276, 33964-33968.

Mayerhofer K, Stozlechner J, Yidiz S, Haider K, Heinzl H, Jakesz R, Pecherstorfer M, Rosen H, Svelda P, Zeillinger R, Speiser P (1996) Plasminogen activator inhibitor 1 and prognosis in breast carcinoma. Geburt Frauenheil 56; 23-27.

Mazzieri R, Masiero L, Zanetta L, Monea S, Onisto M, Garbisa S, Mignatti P (1997) Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulory mechanism with cell-bound reactants. EMBO J 16, 2319-2331.

Okedon LE, Sato Y, Rifkin DB (1992) Urokinase-type plasminogen activator mediates basic growth factor-induced bovine endothelial migration independent of its proteolytic activity. J Cell Physiol 150, 258-263.

Ossowski L, Aguirre-Ghiso JA (2000) Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. Curr Opin Cell Biol 12, 613-620.

Pedersen H, Brunner N, Francis D, Osterlund K, Ronne E, Hansen HH, Dano K, Grondahl-Hansen J (1994) Prognostic impact of urokinase, receptrom and plasminogen activator inhibitor-1 in squamous and large cell lung cancer tissue. Cancer Res 54; 4671-4675.

Pepper MS, Vassalli JD, Montesano R, Orci L (1987) Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. J Cell Biol 105, 2535-2541.

Pepper MS, Sappino AP, Montesano R, Orci L, Vassalli JD (1992) Plasminogen activator inhibitor-1 is induced in migrating capillary endothelial cells. J Cell Physiol 153, 129-139.

Pollanen J, Ross W, Vaheri A (1991) Dirceted plasminogen activatiõn at the surface of normal and malignant cells. Adv Cancer Res 57;272-328.

Providence KM, Kutz SM, Staiano-Coico L, Higgins PJ (2000) PAI-1 gene expression is regionally induced in wounded epithelial cell monolayers and required for injury repair. J Cell Physiol 182; 269-280.

Providence KM, Staiano-Coico L, Higgins PJ (2002) A quantifiable in vitro model to assess the effects of PAI-1 gene targeting on epithelial cell motility. Wound Healing:

Methods and Protocols (Burns-harring A, DiPietro LA, Editors), Human Press, NJ (in press).

Reijerkerk A, Voest EE, Gebbink MF (2000) No grip, no growth: the conceptual basis of excessive proteolysis in the treatment of cancer. Eur J Cancer 36, 1695-1705.

Schmitt M, Janicke F, Graeff H (1992) Tumor-associated proteases. Fibrinolysis 6; 3-26.

Seddighzadeh M, Zhou JN, Kronnwett U, Shoshan MC, Auer G, Sten-Linder M, Wiman B, Linder S (1999) ERK signaling in metastatic human MDA-MB-231 breast carcinoma cells is adapted to obtain high urokinase expression and rapid cell proliferation. Clin Exp Metastasis 17, 649-654.

Stefansson S, Petitclerc E, Wong MK, McMahon GA, Brooks PC, Lawrence DA (2001) Inhibition of angiogenesis in vivo by plasminogen activator inhibitor-1. J Biol Chem 276, 8135-8141.

Torre EA, Fulco RA (1996) Tumor-associated urokinase-type plasminogen activator: significance in breast cancer. Europ J Gynaecol Oncol 17;315-318.

Vaheri A, Stephens RW, Salon EM, Pollanen J, Tapiovaara H (1990) Plasminogen activation at the cell surface-matrix interface. Cell Differen Develop 32; 255-262.

Wysocki AB, Kusakabe AO, Chang S, Tuan TL (1999) Temporal expression of urokinase plasminogen activator, plasminogen activator inhibitor and gelatinase-B in chronic wound fluid switches from a chronic to acute wound profile with progression to healing. Wound Repair Regen 7, 154-165.

APPENDIX

Several manuscripts detailing results of this study in the MCF-7 and MDA-MB-231 clonal isolates are currently in preparation. As these reprints become available they will be forwarded to the USAMRMC

Additional Requested Information

P.I. Contact Data

Paul J. Higgins, Ph.D.
Professor & Director
Center for Cell Biology & Cancer Research
Mail Code 165
Albany Medical College
47 New Scotland Avenue
Albany, NY 12208

Tel: 518-262-5168
Fax: 518-262-5696

E-mail: higginp@mail.amc.edu

List of Personnel Receiving Salary From this Award

Paul J. Higgins, Ph.D.
Lisa A. White, Ph.D.
Jeffrey Boehm, M.S.
Kirwin Providence, M.S.
Jianzhong Tang, M.S.
Rosalie Allen, B.S.

Publications Citing Support From this Award

Manuscripts

Providence KM, Kutz SM, Staiano-Coico L, Higgins PJ (2000) PAI-1 gene expression is regionally induced in wounded epithelial cell monolayers and required for injury repair. **Journal of Cellular Physiology** 182: 269-280.

Lee F, Goncalves J, Faughman K, Steiner MG, Pagan-Charry I, Chin B, Providence KP, Higgins PJ, Staiano-Coico L (2000) Targeted inhibition of wound-induced PAI-1 expression alters migration and differentiation in human epidermal keratinocytes. **Experimental Cell Research** 258: 245-253.

White LA, Bruzdzinski C, Kutz SM, Gelehrter TD, Higgins PJ, (2000) Growth state-dependent binding of USF-1 to a proximal promoter E box element in the rat plasminogen activator inhibitor type-1 gene. **Experimental Cell Research** 260: 127-155.

Kutz SM, Providence KM, Higgins, PJ (2001) Antisense targeting of c-*fos* transcripts inhibits serum-and TGF-β1-stimulated PAI-1 gene expression and directed motility in renal epithelial cells. **Cell Motility and the Cytoskeleton** 48, 163-174.

Kutz SM, Hordines J, McKeown-Longo PJ, Higgins PJ (2001) TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. **Journal of Cell Science** 114, 3905-3914.

Providence KM, Staiano-Coico L, Higgins PJ, (2002) A Quantifiable in vitro model to assess the effects of PAI-1 gene targeting on epithelial cell motility. In: Wound Healing: Methods and Protocols (Burns-Harring A, DiPietro LA, editors), Humana Press, NJ (in press).

Samarakoon R, Higgins PJ (2002) MEK/ERK pathway mediates cell shape-dependent plasminogen activator inhibitor type-1 gene expression upon drug-induced disruption of the microfilament and microtubule networks. **Journal of Cell Science** (accepted for publication).

Providence KM, White CA, Tang J, Gonclaves J, Staiano-Coico L, Higgins PJ (2002) Epithelial monolayer wounding stimulates binding of USF-1 to an E box motif in the Plasminogen Activator Inhibitor Type-1 gene. **Journal of Cell Science** (accepted for publication).

Higggins PJ (2002) PAI-1 expression is elevated in aggressive breast carcinoma cells and required for tissue invasion. **Journal of Cellular Physiology** (submitted for publication).

Abstracts

Ozsvath KJ, Kutz SM, Shah, DM, Higgins PJ (1999) Requirements for PAI-1 gene expression in tubulogenic T2 rat cell lines. **Proceedings of the Society for Vascular Surgery**, 15th Annual Vascular Fellows Abstract Presentation.

Higgins PJ (2000) PAI-1 gene expression in breast carcinoma cells: implications for cellular migratory activity. **Proceedings of the Department of Defense Breast Cancer Research Meeting,** I, 85.

Higgins PJ (2000) Antisense targeting of PAI-1 expression inhibits TGF-β1-induced migration in premalignant epidermal epithelial cells. **"New Molecular Targets for Cancer Therapy" Conference Proceedings.**

Kutz SM, Higgins PJ (2001) TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. **Molecular Biology of the Cell** 12, 281a.

Samarakoon R, Higgins PJ (2001) Cytoskeletal controls on PAI-1 gene regulation. **Molecular Biology of the Cell** 12, 353a-354a.

Higgins PJ, Tang, J (2001) PAI-1 promoter-driven PAI-1-GFP expression vectors for direct assessment of transcriptional activity in individual cells *in vivo.* **Proceedings of the American Association for Cancer Research Special Conference on "Molecular Imaging in Cancer: Linking Biology, Function, and Clinical Applications *In Vivo,*** p. A-8.

Higgins PJ (2002) PAI-1 is an important regulator of substrate adhesion and migration in human breast carcinoma cells. **Proceedings of the Department of Defense Breast Cancer Research Meetings.**

Higgins PJ (2002) PAI-1 promoter-driven PAI-1-GFP expression vectors for *in vitro* assessment of transcriptional activity in individual cells**. Proceedings of the Second Biennial Meeting: Molecular Targets for Cancer Therapy**.

Higgins PJ (2002) PAI-1 promoter-driven PAI-1-GFP expression vectors: tools for assessment of transcriptional activity and cellular invasive traits. **Proceedings of the XXXth meeting of the International Society for Oncodevelopmetnal Biology and Medicine**.

Lectures

(1999) *"PAI-1 Gene Regulation and Cell Transformation"*. Endocrinology and Cancer Seminar Program, David Axelrod Institute, Albany, New York, April 3.

(2000) *"Molecular Mechanisms Underlying Growth State-Dependent PAI-1 Gene Expression"*. Stratton VA Medical Center Research Seminar, Albany, New York, February 17.

(2000) *"Genetic Targeting of PAI-1 Gene Expression: Consequences on Cell Migration"*. David Axelrod Institute, Cancer and Endocrine Signaling Seminar Series, Albany, New York, march 13.

(2001) *"Molecular Mechanisms of PAI-1 Gene Control: Novel Targets for Tumor Therapy"*. Mayo Clinic Cancer Center, Jacksonville, Florida, November 15.

(2002) *"PAI-1 Gene Targeting: Consequences on Cell Migration"*. Stratton VA Medical Center Research Seminar, Albany, New York, April 25.

Table 1. Migratory characteristics of parental and transfectant human breast carcinoma cell lines.

Cell Line	Relative PAI-1 Expression	Migration Indices Chemokinetic	Chemotactic
MCF-7	1.0	276±129	366±109
7/CMV	0.8±0.1	201±74	290±59
7/EBPAI-D	4±1	297±101	431±207
7/CMVPAI-E	12±3	432±88	716±244
7/CMVPAI-B	22±5	699±121	701±153
7/CMVPAI-K	30±6	748±97	1289±346
7/CMVPAI-H	49±9	1121±277	3348±601
MDA-MB-231	100	742±93	2392±316
231/CMV	108±11	891±76	2203±237
231/CMVIAP-6	89±20	525±43	1114±179
231/CMVIAP-9	64±7	312±44	700±191
231/EBIAP-15	40±9	285±59	513±105
231/EBIAP-3	17±6	171±22	327±98
231/EBIAP-21	8±3	143±6	202±53

Relative PAI-1 Expression for MCF-7 transfectants = fold increase in de novo synthesized PAI-1 protein relative to parental cells (set at 1.0).

Relative PAI-1 Expression for 231 transfectants = % decrease in de novo synthesized PAI-1 protein relative to parental cells (set at 100%).

Migration Indices for the chemokinetic (without attractant gradient) and chemotactic (with attractant gradient) indicates the number of cells migrating to the lower chamber of a micro-chemotaxis apparatus within 3 hours after seeding of 5×10^4 cells to the upper chamber.

Data expressed = mean±standard deviation

Table 2. Effect of PAI-1 and PAI-1 neutralizing antibodies on adhesive and migratory characteristics of MDA-MB-231 human breast carcinoma cells.

Treatment	Adhesion[1]	2-D Migration[2]	Invasion[3]
None	100	100	100
[4]PAI-1 (1μg)	136±9	127±6	143±11
PAI-1 (5μg)	166±13	139±8	162±15
[5]PAI-1P14 (1μg)	179±26	144±14	133±17
PAI-1P14 (5μg)	202±34	151±18	189±22
[6]control IgG	98±4	96±5	102±8
PAI-1 nIgG	15±3	47±10	30±6

[1] Adhesion represents the % cells that attach and spread on a tissue culture plate within 2 hours after seeding in 10% FBS-containing medium relative to non-treated control cells (control value=100%)

[2] Planar migration rate relative to control cells as measured 24 hours post-scrape injury (method of Providence et al., 2000)

[3] Fraction (% of control) of cells that migrate to the bottom of Matrigel-coated transwell filters 24 hours after seeding to the top chamber.

[4] Wild-type recombinant PAI-1 used at 1 or 5 μg/ml.

[5] Stabilized recombinant PAI-1 (PAI-1P14; H10R, N150H, K154T, Q319L, M354I, T333R mutant) used at 1 or 5 μg/ml.

[6]IgG control or neutralizing IgG (nIgG) used at 5 μg/ml.

Data expressed = mean±standard deviation

Table 3. Migratory characteristics of MDA-MB-231 transfectant cells expressing a PAI-1-GFP fusion protein.

Cell Type	Migration[3]	Invasion[4]
Control	100	100
GFP transfectants[1]	$98^{\pm}3$	$94^{\pm}8$
PAI-1-GFP transfectants[2]	$139^{\pm}12$	$163^{\pm}37$

1. Cell transfected with a GFP-producing vector only.

2. Cells transfected with the chimeric PAI-1-GFP expression vector (Figure 1)

3. 2-D planar migration rate relative to control cells (i.e., untransfected parental stock) as determined 24 hours post-scrape injury (method of Providence et al; 2000).

4. Fraction (% of control) of cells that migrate to the bottom of Matrigel-coated transwell filters 24 hours after seeding to the top chamber.

Data expressed = means $^{\pm}$ standard deviation

Tabel 4. Tumorigenicity of MDA-MB-231 transfectants

Cell Line	Tumor Incidence[1]	Lung Nodules[2]
MDA-MB-231	2/2 (1356)	2/2
231/CMV	2/2 (1191)	2/2
231/CMVIAP-6	3/3 (1262)	2/3
231/CMVIAP-9	2/3 (874)	2/3
231/EBIAP-15	3/3 (713)	1/3
231/EBIAP-3	2/3 (557)	0/2
231/EBIAP-21	3/3 (412)	0/3

1. Incidence of tumors formed upon inoculation of $3x10^6$ cells subcutaneously in the region of the mammary fat pad. The number in parenthesis indicates the mean tumor volume (mm^3) calculated at 10 weeks post-injection.

2. Incidence of one or more grossly visible lung nodules in mice that formed tumors upon transplantation with the indicated cell type.

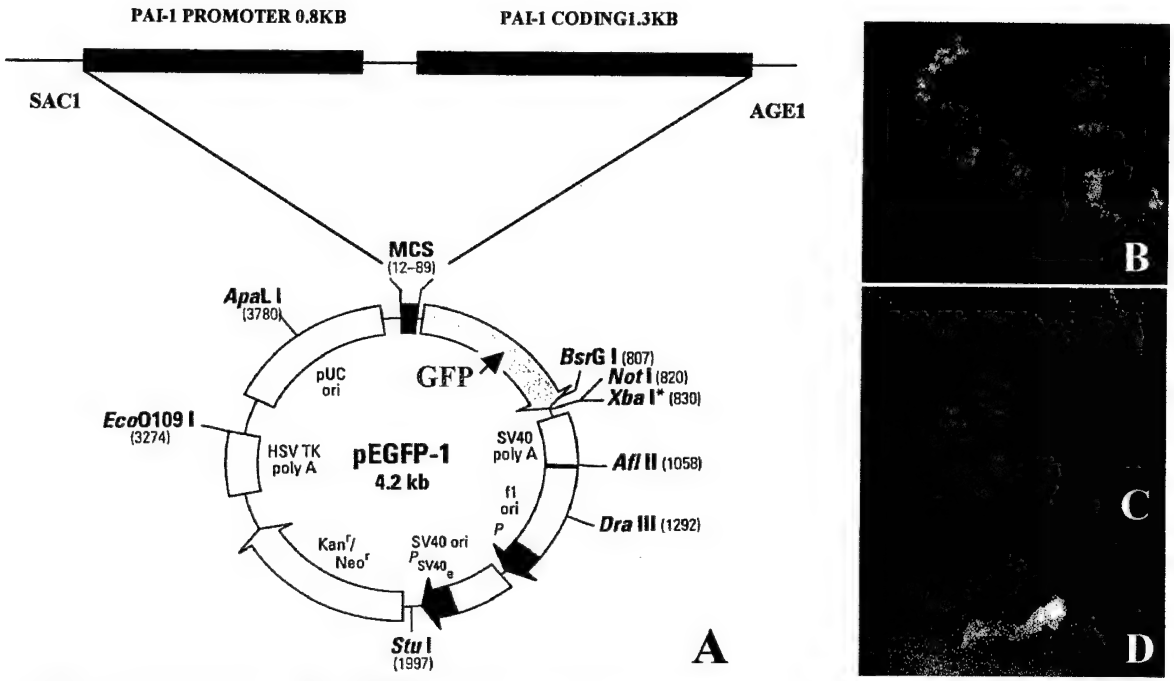

Figure 1. Visualization of PAI-1-GFP in cellular migration tracks. Schematic of a pEGFP-1-based vector in which a chimeric transcript consisting of 1.3 kb of PAI-1 coding sequences and GFP is expressed under the control of a 0.8 kb PAI-1 promoter **(A)**. Transfection of epithelial cells with thi.s reporter results in initial accumulation of PAI-1-GFP in the golgi **(B)** followed by deposition into the extracellular matrix and finally into cellular migration "trails" **(B,C)**

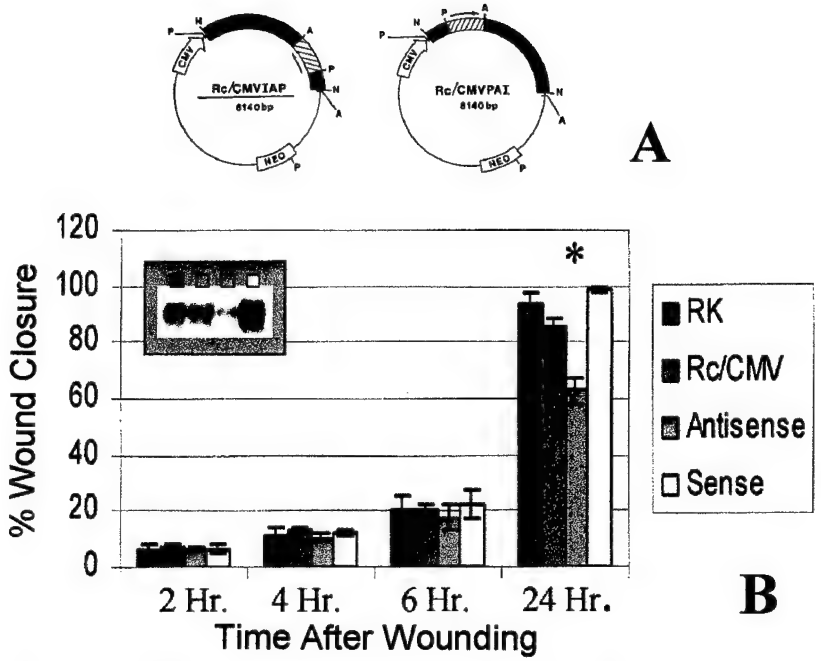

Figure 2. Effect of targeted PAI-1 down-regulation on cell motility. RK epithelial cells were transfected with the indicated constructs **(A)**, grown to confluence and scrape-wounded. The rate of wound closure **(B)** is a measure of migratory ability in this system and is significantly impaired by PAI-1 down-regulation as determined by Western blotting of cell extracts at the 24 hour time point (insert in **B**).

TGF-β1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion

Stacie M. Kutz, John Hordines, Paula J. McKeown-Longo and Paul J. Higgins*

Center for Cell Biology & Cancer Research, Albany Medical College, Albany, New York 12208, USA

*Author for correspondence (e-mail: higginp@mail.amc.edu)

Accepted 16 July 2001
Journal of Cell Science 114, 3905-3914 (2001)

SUMMARY

The type-1 inhibitor of plasminogen activator (PAI-1) is an important physiological regulator of extracellular matrix (ECM) homeostasis and cell motility. Various growth factors mediate temporal changes in the expression and/or focalization of PAI-1 and its protease target PAs, thereby influencing cell migration by barrier proteolysis and/or ECM adhesion modulation. TGF-β1, in particular, is an effective inducer of matrix deposition/turnover, cell locomotion and PAI-1 expression. Therefore, the relationship between motility and PAI-1 induction was assessed in TGF-β1-sensitive T2 renal epithelial cells. PAI-1 synthesis and its matrix deposition in response to TGF-β1 correlated with a significant increase in cell motility. PAI-1 expression was an important aspect in cellular movement as PAI-1-deficient cells had significantly impaired basal locomotion and were unresponsive to TGF-β1. However, the induced migratory response to this growth factor was complex. TGF-β1 concentrations of 1-2 ng/ml were significantly promigratory, whereas lower levels (0.2-0.6 ng/ml) were ineffective and final concentrations ≥5 ng/ml inhibited T2 cell motility. This same growth factor range progressively increased PAI-1 transcript levels in T2 cells consistent with a bifunctional role for PAI-1 in cell migration. TGF-β1 induced PAI-1 mRNA transcripts in quiescent T2 cells via an immediate-early response mechanism. Full TGF-β1-stimulated expression required tyrosine kinase activity and involved MAPK/ERK kinase (MEK). MEK appeared to be a major mediator of TGF-β1-dependent PAI-1 expression and T2 cell motility since PD98059 effectively attenuated both TGF-β1-induced ERK1/2 activation and PAI-1 transcription as well as basal and growth factor-stimulated planar migration. Since MEK activation in response to growth factors is adhesion-dependent, it was important to determine whether cellular adhesive state influenced TGF-β1-mediated PAI-1 expression in the T2 cell system. Cells maintained in suspension culture (i.e., over agarose underlays) in growth factor-free medium or treated with TGF-β1 in suspension expressed relatively low levels of PAI-1 transcripts compared with the significant induction of PAI-1 mRNA evident in T2 cells upon stimulation with TGF-β1 during adhesion to a fibronectin-coated substrate. Attachment to fibronectin alone (i.e., in the absence of added growth factor) was sufficient to initiate PAI-1 transcription, albeit at levels considerably lower than that induced by the combination of cell adhesion in the presence of TGF-β1. T2 cells allowed to attach to vitronectin-coated surfaces also expressed PAI-1 transcripts but to a significantly reduced extent relative to cells adherent to fibronectin. Moreover, newly vitronectin-attached cells did not exhibit a PAI-1 inductive response to TGF-β1, at least during the short 2 hour period of combined treatment. PAI-1 mRNA synthesis in response to substrate attachment, like TGF-β1-mediated induction in adherent cultures, also required MEK activity as fibronectin-stimulated PAI-1 expression was effectively attenuated by the MEK inhibitor PD98059. These data indicate that cellular adhesive state modulates TGF-β1 signaling to particular target genes (i.e., PAI-1) and that MEK is a critical mediator of the PAI-1$^+$/promigratory phenotype switch induced by TGF-β1 in T2 cells.

Key words: PAI-1, Gene expression, Signal transduction, TGF-β1

INTRODUCTION

Genetic analysis and adaptation of physiologically-relevant in vitro models of wound repair have clarified basic mechanisms involved in the tissue response to injury (Garlick and Taichman, 1994; Romer et al., 1996; Creemers et al., 2000; Providence et al., 2000). Fundamental to this process is the conversion of normally sedentary cells to an actively migrating, invasive phenotype (Martin, 1997). However, stimulated cell movement and locomotion through the provisional extracellular matrix (ECM) requires cycles of adhesion-deadhesion and precise control of the pericellular proteolytic environment (Yamada and Clark, 1996; Greenwood and Murphy-Ullrich, 1998; Xie et al., 1998; Pilcher et al., 1999). Efficient wound re-epithelialization involves several protease systems with repair outcome highly dependent on the generation of plasmin by urokinase plasminogen activator (uPA). uPA activity is regulated, in turn, by its fast-acting type-1 inhibitor (PAI-1) (Andreasen et al., 1997; Lund et al., 1999; Zhou et al., 2000; Legrand et al., 2001). This cascade directly influences the overall injury site proteolytic balance and is a critical determinant in wound resolution (Mazzieri et al., 1997; Wysocki et al., 1999) as well as directed cell movement (Pepper et al., 1987; Pepper et al., 1992; Okedon et al., 1992; Providence et al., 2000).

Injury-induced cell motility is orchestrated by various

autocrine/paracrine-acting growth factors (Martin, 1997). Most prominent are members of the transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), and epidermal growth factor (EGF) families (Boland et al., 1996; Sato and Rifkin, 1988; Song et al., 2000; Ellis et al., 2001; Goke et al., 2001). TGF-β1 and activin A, in particular, integrate the complex processes of tissue repair and cell migration (Zambruno et al., 1995; Munz et al., 1999) largely through control of genes that encode matrix components (fibronectin, type I collagen), regulators of ECM homeostasis (e.g., uPA, PAI-1) and the cellular adhesive apparatus (e.g., PAI-1, integrin subunits) (Cajot et al., 1989; Cajot et al., 1990; Wrana et al., 1991; Munz et al., 1999; Lai et al., 2000; Providence et al., 2000). Therefore, growth factor-initiated changes in the expression, focalization and/or relative activity of uPA/PAI-1 may stimulate or inhibit cell migration via ECM barrier proteolysis or by altering cellular adhesive interactions with the ECM (Stefansson and Lawrence, 1996; Mignatti and Rifkin, 2000). Variances in PAI-1 synthesis (Providence et al., 2000) and/or site-localization (Kutz et al., 1997) would specifically impact on cellular migration by affecting uPA activity as well as uPAR/vitronectin- or integrin/vitronectin-dependent cell attachment (Ciambrone and McKeown-Longo, 1990; Deng et al., 1996; Chapman, 1997; Stefansson and Lawrence, 1996; Loskutoff et al., 1999).

Since TGF-β1 stimulates cell motility (Kutz et al., 2001), PAI-1 induction (Boehm et al., 1999) in response to TGF-β1 is probably critical to the motile process and the acquisition of epithelial cell 'plasticity' (Akiyoshi et al., 2001; Zavadil et al., 2001). This was confirmed in the present study using the PAI-1-deficient 4HH cell line (Providence et al., 2000) in a quantitative model of induced cell locomotion. Therefore, it was important to define mechanisms involved in TGF-β1-dependent PAI-1 gene expression. PAI-1 transcription in TGF-β1-responsive T2 epithelial cells used an immediate-early, tyrosine kinase-mediated, signaling pathway. Moreover, PAI-1 induction and basal as well as TGF-β1-stimulated T2 cell locomotion was MEK-dependent. The involvement of MEK in TGF-β1-initiated PAI-1 expression and the adhesion-dependency of MEK activation (Renshaw et al., 1997) suggested that substrate attachment may influence TGF-β1-induced PAI-1 gene regulation. TGF-β1, in fact, poorly induced PAI-1 transcription in cells maintained in suspension culture but significantly increased PAI-1 expression during attachment to fibronectin-coated surfaces. Cellular adherence to fibronectin alone (i.e., in the absence of TGF-β1), and to a lesser extent vitronectin, also stimulated PAI-1 mRNA synthesis indicating that adhesive state can modulate TGF-β1 signaling to particular target genes (i.e., PAI-1).

MATERIALS AND METHODS

Cell culture

T2 and EC-1 renal epithelial cells and the PAI-1-deficient 4HH line (Providence et al., 2000) were cultured in DMEM containing 10% fetal bovine serum (FBS) and antibiotics. Cells were washed twice with Hanks' balanced salt solution (HBSS; 1.3 mM $CaCl_2$, 5 mM KC1, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$-$6H_20$, 0.4 mM $MgSO_4$-$7H_20$, 0.14 m NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose) then incubated in serum-free DMEM for 3 days to initiate a quiescent state (Kutz et al., 1997). Cells were either maintained in quiescence medium or stimulated by the direct addition of FBS or TGF-β1 (to final concentrations of 20% and 0.2-10.0 ng/ml, respectively). Puromycin (100 μg/ml), actinomycin D (5 μg/ml), genistein (100 μM), PD98059 (5-50 nM) or wortmanin (50 nM) were added 30 minutes prior to stimulation with FBS or TGF-β1. For culture under non-adherent conditions, quiescent T2 cells were harvested by trypsinization, washed with soybean trypsin inhibitor, and plated on 1% agarose underlays in serum-free DMEM for 4 hours (Ryan et al., 1996). Agarose-suspended cells were maintained (for an additional 2 hours) under non-adherent conditions (in the presence or absence of TGF-β1) or seeded onto fibronectin-, vitronectin- or bovine serum albumin (BSA)-coated 100 mm plastic dishes (coating concentration 10 μg protein/ml) and allowed to re-attach for 2-6 hours (in the presence or absence of TGF-β1) prior to RNA isolation. Directional migration assays used methods developed previously (Providence et al., 2000). Three-day post-confluent T2 cell cultures were washed twice with HBSS and incubated for 3 days in serum-free DMEM. Monolayers were maintained under serum-free or TGF-β1-/serum-supplemented conditions prior to scrape-wounding using the small end of a 1000 μl pipette tip. Initial wound size was determined for each culture dish and extent of injury closure assessed 24 hours later with an inverted microscope fitted with a calibrated ocular grid.

Northern blot analysis

Total cellular RNA was isolated and denatured at 55°C for 15 minutes in 1× MOPS, 6.5% formaldehyde and 50% formamide prior to electrophoresis on agarose/formaldehyde gels (1.2% agarose, 1.1% formaldehyde, 1× MOPS, 50 mM sodium acetate, 1 mM EDTA, pH 8.0). RNA was transferred to Nytran membranes by capillary action in 10× SSC (3M NaCl, 0.3 M Na citrate, pH 7.0), UV crosslinked and incubated for 2 hours at 42°C in 50% formamide, 5× Denhardt's solution, 1% SDS, 100 μg/ml sheared/heat-denatured salmon sperm DNA (ssDNA) and 5× SSC. RNA blots were hybridized with ^{32}P-labeled cDNA probes for PAI-1 and A-50 (Ryan and Higgins, 1993) for 24 hours at 42°C in 50% formamide, 2.5× Denhardt's solution, 1% SDS, 100 μg/ml ssDNA, 5× SSC and 10% dextran sulfate. Membranes were washed 3 times in 0.1× SSC/0.1% SDS for 15 minutes each at 42°C followed by 3 washes at 55°C prior to exposure to film.

Microscopy

Cells were washed twice with Ca^{2+}/Mg^{2+} free phosphate-buffered saline (PBS-CMF; 2.7 mM KC1, 1.2 mM KH_2PO_4, 0.14 M NaC1, 8.1 mM Na_2HPO_4-$7H_2O$) and fixed in 10% formalin/PBS-CMF for 10 minutes. Following permeabilization with cold 0.5% Triton X-100/PBS-CMF (for PAI-1 immunolocalization) or 1% NP-40/PBS-CMF (for phalloidin-actin binding) for 10 minutes at 4°C, cells were washed 3 times (5 minutes each) with PBS-CMF then overlayed with rabbit antibodies to PAI-1 (Kutz et al., 1997) in BSA (3 mg/ml)/PBS-CMF. After three PBS-CMF washes, cells were incubated with fluorescein-conjugated goat anti-rabbit IgG (1:20 in BSA/PBS-CMF) for 30 minutes at 37°C, washed, and coverslips mounted with 100 mM *n*-propylgalate in 50% glycerol/PBS-CMF. Rhodamine-phalloidin was used to visualize actin microfilament structures (Ryan and Higgins, 1993).

MAP kinase assays

Cells were extracted for 30 minutes in cold lysis buffer (0.5% deoxycholate, 50 mM Hepes [pH 7.5], 1% Triton X-100, 1% NP-40, 150 mM NaCl, 50 mM NaF, 1 mM Na-orthovanadate, 0.1% aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF) and lysates clarified by centrifugation at 14,000 ***g*** for 15 minutes at 4°C. For immunoprecipitation, aliquots containing 500 μg protein were incubated with 2 μg ERK1/2 antibody for 2 hours with gentle rocking. Protein A/G Plus-agarose (30 μl) was added for 2 hours, immune complexes collected by centrifugation, washed twice with lysis buffer and twice with 100 mM NaCl in 50 mM Hepes (pH 8.0) and then

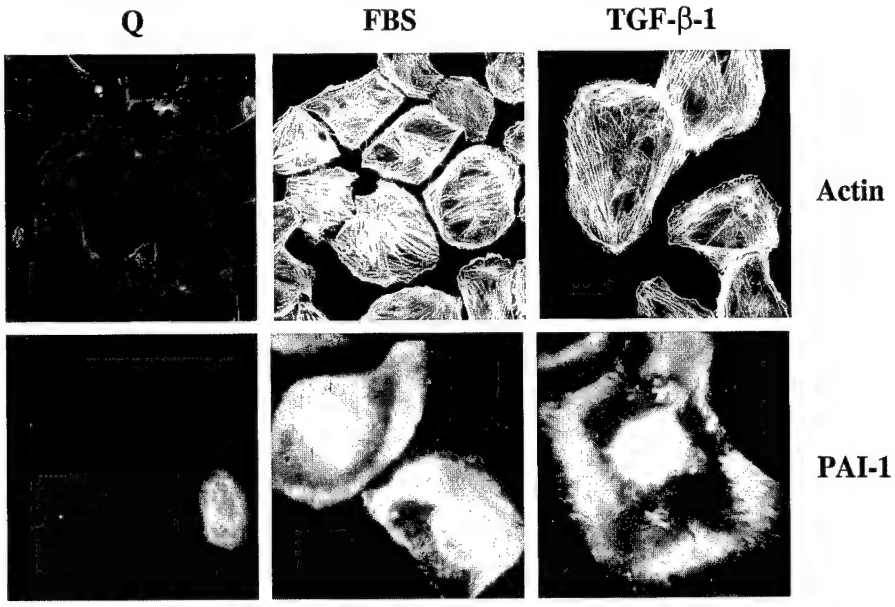

Fig. 1. PAI-1 deposition in T2 cells after stimulation with serum or TGF-β1. Quiescent (Q) cell cultures were stimulated by addition of FBS or TGF-β1 (to final concentrations of 20% and 1 ng/ml, respectively). After 4 hours, cells were fixed and processed (see Materials and Methods) for visualization of microfilament organization (Actin) and PAI-1 immunolocalization (PAI-1).

incubated at 37°C for 15 minutes in kinase reaction buffer (10 μCi ^{32}P-ATP, 50 μM ATP, 20 mM Hepes (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine, 0.3 mg/ml myelin basic protein (MBP)). Electrophoresis buffer (50 mM Tris (pH 6.0), 10% glycerol, 1% SDS, 1% β-mercaptoethanol) was added, samples boiled for 10 minutes and 15 μl aliquots separated on SDS/15% polyacrylamide gels. Proteins were transferred to nitrocellulose in 25 mM Tris, 190 mM glycine, 20% methanol and membranes exposed to film for visualization of phosphorylated MBP. Western blotting for ERK2 and total MBP detection by Ponceau S staining were used to confirm equivalent loading per lane. For detection of phosphorylated ERK1/2, membranes were washed for 10 minutes in 0.05% Triton X-100/PBS-CMF followed by 2 hours in wash solution containing 3% milk. Phospho-ERK monoclonal antibody (1:1000) was added for an overnight incubation in blocking solution at room temperature. Following 3 washes for 20 minutes, horseradish peroxidase (HRP)-labeled anti-mouse secondary antibody (1:3000 in blocking solution) was added and incubated for an additional 1 hour. Membranes were washed 5 times for 10 minutes each in wash solution, incubated with ECL substrate solution (Amersham, Piscataway, NJ) for 2 minutes with gentle rocking and exposed to film. Membranes were stripped for 90 minutes at room temperature using the Western Stripper Kit (Bioworld, Dublin, Ohio), neutralized then incubated in a ERK1/2 primary antibody mixture (each diluted 1:3000 in blocking solution) followed by HRP-anti-rabbit secondary antibody and ECL reagent as described above.

RESULTS

TGF-β1 stimulation of PAI-1 expression and directional motility is MEK-dependent

PAI-1 synthesis and accumulation in the ventral undersurface region in response to TGF-β1 (Fig. 1) correlated with a significant increase in T2 cell motility (relative to the basal rate of movement in the scrape-wound assay) approximating that of serum-stimulated cells (Fig. 2). This rather dramatic effect of TGF-β1 on cell migration and cytoarchitecture is consistent with involvement of TGF-β1 target genes (e.g., PAI-1) in cellular 'plasticity' and invasive behavior (Zavadil et al., 2001). Antibodies to PAI-1, in fact, inhibit cell attachment (Higgins et al., 1991), promote substrate detachment (Rheinwald et al., 1987) and attenuate cell activation (Kutz et al., 1997). Furthermore, recent findings have highlighted the functional linkage between induced PAI-1 synthesis and cellular motility (Mignatti and Rifkin, 2000; Providence et al., 2000; Kutz et al., 2001). To directly assess the role of PAI-1 in growth factor-stimulated cell locomotion, TGF-β1-dependent wound closure was compared in EC-1 and 4HH cells. 4HH is a derivative of EC-1 in which PAI-1 synthesis is specifically ablated by stable constitutive expression of PAI-1 antisense transcripts under

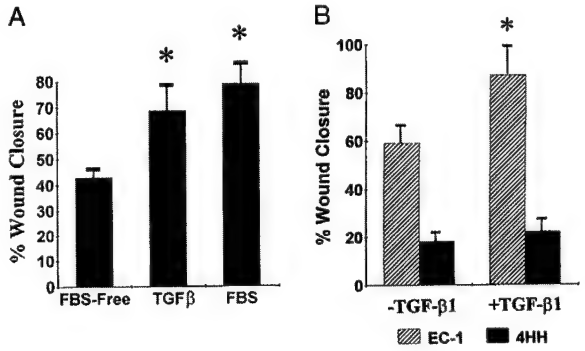

Fig. 2. Stimulation of cell motility by serum or TGF-β1. Confluent cultures of T2 cells were incubated in serum-free DMEM for 3 days prior to scrape-wounding. Cells were maintained in the FBS-free medium to assess basal migration (A). The percent (%) wound closure was determined 24 hours later. Data plotted are the means±s.d. of 20 individual measurements made on each of triplicate cultures for each treatment condition. Asterisks indicate a statistically significant difference (Student's *t*-test, $P>0.01$) in cell migration for TGF-β1- and FBS-supplemented cultures compared with basal (FBS-free) motility. The effect of targeted PAI-1 ablation on basal (–TGF-β1) as well as TGF-β1-induced (+TGF-β1) cell locomotion was assessed in EC-1 and 4HH cell cultures by evaluation of the extent (%) of wound closure in the absence and presence of growth factor (1 ng/ml) (B). Data plotted are the means±s.d. of 15 individual measurements on duplicate cultures/treatment group. Asterisk indicates a statistically significant difference ($P<0.01$) between motility under growth factor-free and supplemented conditions for EC-1 cells. By contrast, 4HH cells were unresponsive to TGF-β1 in this assay.

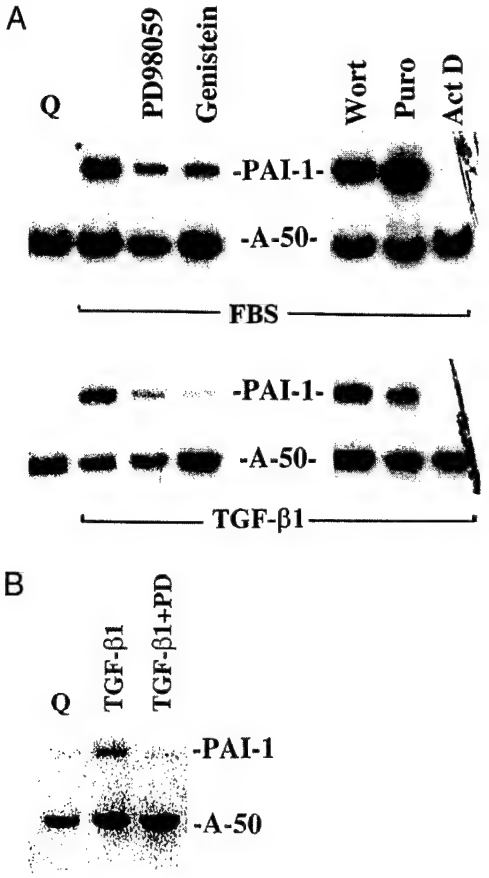

Fig. 3. Metabolic requirements for TGF-β1-induced PAI-1 expression. To assess pathways underlying induced PAI-1 expression, quiescent (Q) T2 cells were stimulated with serum (20%) or TGF-β1 (1 ng/ml) for 2 hours, in the presence or absence of a 30 minute pretreatment with the indicated inhibitors, prior to RNA isolation (A). Northern blots were hybridized with ^{32}P-labeled cDNA probes for PAI-1 and A-50 simultaneously. The inability of puromycin to attenuate either serum or TGF-β1-induced PAI-1 transcripts and the sensitivity of expression to actinomycin D indicated that PAI-1 induction by both stimuli was an immediate-early (i.e., primary) response. TGF-β1-induced PAI-1 expression in T2 cells is MEK-dependent (B). Quiescent (Q) T2 cells were stimulated with TGF-β1 (1 ng/ml) for 2 hours in the absence or presence of a 30 minute pretreatment with PD98059 (50 nM) prior to isolation of RNA. Northern blots were hybridized with ^{32}P-labeled cDNA probes to PAI-1 and A-50.

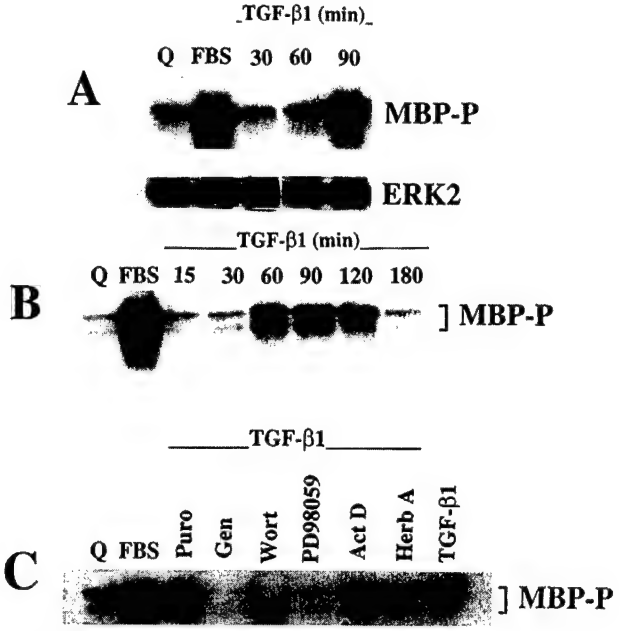

Fig. 4. Coupled ERK immunoprecipitation/MBP kinase assay for assessment of TGF-β1-induced ERK activation. ERK1/2 were immunoprecipitated from lysates of quiescent (Q), FBS- and TGF-β1-stimulated T2 cells. Exposure of quiescent cells to FBS (20%) was for 15 minutes and stimulation with TGF-β1 (1 ng/ml) was for 30, 60 and 90 minutes prior to cell disruption and ERK1/2 immunoprecipitation. MBP phosphorylation reaction products (MBP-P) were separated by gel electrophoresis; equivalent loading of MBP and ERK per lane was confirmed by Ponceau S staining (not shown) and ERK2 western blotting, respectively (A). In contrast to the relatively rapid rate of ERK activation by serum (15 minutes), TGF-β1-induced changes in ERK activity were not evident until 60 minutes after growth factor addition (A), remained elevated for approximately 2 hours and then rapidly declined (B). Coupled ERK immunoprecipitation/MBP phosphorylation (MBP-P) assays (C) confirmed that ERK activation in TGF-β1-stimulated T2 cells is sensitive to the same pharmacologic inhibitors that attenuate growth factor-induced PAI-1 expression. The more pathway restrictive inhibitor herbimycin A (250 nM) (Fukazawa et al., 1994) attenuated MBP phosphorylation but not to the same extent as genistein or PD98059.

control of a strong CMV promoter (Higgins et al., 1997). These cells do not produce detectable PAI-1 protein under growth factor-supplemented culture conditions (Providence et al., 2000), thus, providing a tool to assess the relationship between PAI-1 expression and cell motility. The 4HH basal migration rate (i.e., locomotion in serum-/growth factor-free medium) was ≤30% that of parental controls. Moreover, relative to EC-1 cells in which wound closure is significantly enhanced by TGF-β1, 4HH cells were unresponsive and failed to increase substantially their rate of movement in TGF-β1-supplemented medium (Fig. 2).

Since PAI-1 appears to be a critical element in cellular migration, it was important to clarify pathways by which TGF-β1 influenced PAI-1 gene expression and the motile phenotype. TGF-β1-mediated PAI-1 transcription, similar to induction by serum, was an immediate-early response (i.e., resistant to protein synthesis inhibitors) and significantly reduced by prior exposure to genistein (Fig. 3). The MEK-specific compound PD98059 effectively attenuated (at the 5-20 nM range) (Fig. 3A) and completely ablated (at 50 nM) (Fig. 3B) TGF-β1-induced PAI-1 expression. In agreement with previous studies (Hartsough and Mulder, 1995; Yonekura et al., 1999), and the PD98059-sensitivity of PAI-1 induction (Fig. 3), addition of TGF-β1 to quiescent T2 cells stimulated ERK1/2 activity. However, the time course of ERK activation by TGF-β1 (as assessed by the ability of ERK1/2 to phosphorylate the target substrate MBP in a linked immunoprecipitation-in vitro kinase assay), was delayed (by approximately 60 minutes) compared with the rapid induction (within 15 minutes) typical of serum-treated cells (Fig. 4). Similarly, increases in phospho-ERK1/2 levels (sixfold those of unstimulated cells) were not evident until 1 hour after addition of TGF-β1 to quiescent cultures (not shown). Consistent with

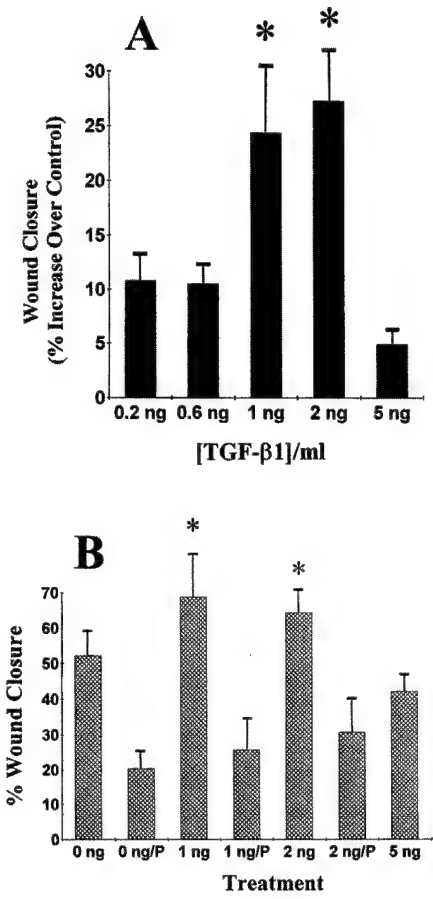

Fig. 5. The MEK inhibitor PD98059 attenuates both basal and TGF-β1-stimulated T2 cell migration. Initial experiments were designed to determine the optimal concentration of TGF-β1 on wound-induced motility (A). After scrape-injury, TGF-β1 was added (in the concentrations indicated) and extent of migration determined 24 hours later. Data plotted is % increase in wound closure relative to non-supplemented (FBS-free) cultures. Asterisks indicate those concentrations for which motility was significantly different from basal migration (Student's *t*-test, $P>0.0005$). To assess the requirement for MEK activity in stimulated cell movement, monolayer scrape wound-closure assays were carried out in TGF-β1-supplemented (concentration range 0, 1, 2 and 5 ng/ml) serum-free medium in the presence (P) or absence of PD98059 (50 μM) (B). TGF-β1 at 1 and 2 ng/ml significantly increased T2 cell directional motility (Student's *t*-test, $P>0.001$; asterisks) relative to basal mobility (0 ng). In this series of experiments, cells exposed to 5 ng/ml of the growth factor actually had a decreased rate of locomotion relative to unsupplemented controls. At each concentration of TGF-β1, PD98059 effectively reduced wound closure; there was no significant difference in the % closure rate among any of the treatment groups in the presence of PD98059.

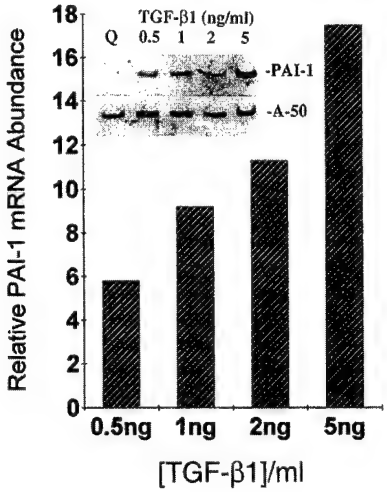

Fig. 6. TGF-β1 concentration-dependent increase in relative PAI-1 mRNA transcripts. Quiescent T2 cells (Q) were stimulated with TGF-β1 at the indicated concentrations and RNA isolated 2 hours later. Northern blots (insert for example) were scanned and the average PAI-1 transcript abundance, normalized to A-50 signal, calculated for 2 separate experiments.

the metabolic requirements for PAI-1 expression in response to TGF-β1 (Fig. 3), genistein as well as PD98059 effectively blocked TGF-β1-mediated ERK1/2 activation in coupled immunoprecipitation/MBP-phosphorylation assays (Fig. 4).

Although MEK was an important intermediate in TGF-β1-dependent PAI-1 transcription, the role of this signaling pathway in TGF-β1-stimulated T2 cell motility was unclear (Kutz et al., 2001). Since the migration-promoting effects of TGF-β1 are often concentration, as well as context-dependent, it was necessary to define the optimal level of growth factor required to maximally stimulate directional T2 cell locomotion. Dose-assessments indicated that the migratory response to TGF-β1 was complex. Motility rates in cultures supplemented with low (0.2-0.6 ng/ml) as well as high (5 ng/ml) TGF-β1 concentrations were not significantly different from control values compared with the obvious promigratory effect associated with exposure to 1 and 2 ng/ml (Fig. 5A). TGF-β1 levels >5 μg/ml actually inhibited wound-induced T2 cell locomotion (when used alone or in the presence of serum) (not shown). PAI-1 transcripts progressively increased over the same growth factor concentration range (0.5-5.0 ng/ml) (Fig. 6). Collectively, these data are consistent with the suggestion that PAI-1 is a bifunctional regulator of cellular motility with positive effects likely restricted to a relatively narrow expression level 'window' (Mignatti and Rifkin, 2000). Using the determined optimal TGF-β1 concentration (1-2 ng/ml), as well as a subeffective 5 ng/ml dose, MEK activity appeared to be important to both TGF-β1-stimulated PAI-1 gene expression and planar motility, as PD98059 effectively attenuated both responses (Fig. 3; Fig. 5B).

Adhesive controls on TGF-β1-induced PAI-1 expression in T2 cells

PD98059 blockade implicated MEK as a critical intermediate in the TGF-β1-initiated pathway of PAI-1 gene expression (Fig. 3). Growth factor- and integrin-activated signaling pathways are interdependent, often converging on the MAPK cascade (Zhu and Assoian, 1995; Lin et al., 1997; Roovers et al., 1999), and MEK activation in response to growth factors requires substrate adhesion (Renshaw et al., 1997). Therefore, experiments were designed to assess whether cellular adhesive state modulated TGF-β1-induced PAI-1 expression. Quiescent T2 cells were trypsinized and plated over agarose underlays in serum-/TGF-β1-free DMEM where they remained in suspension as single cells. After 3 hours, agarose-cultured cells

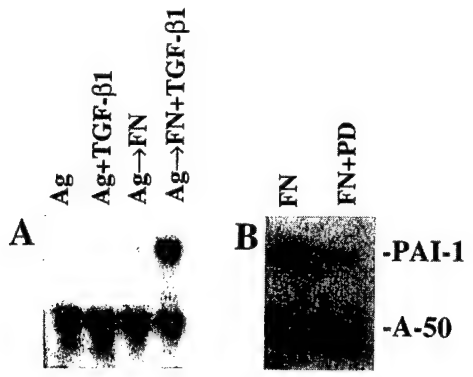

Fig. 7. Optimum response of the PAI-1 gene to TGF-β1 stimulation in T2 cells requires substrate adhesion. TGF-β1-induced PAI-1 transcripts requires adhesion (to a fibronectin (FN) substrate) since cells cultured in suspension (agarose, Ag) or stimulated with TGF-β1 (1 ng/ml) in suspension (Ag+TGF-β1) did not express PAI-1 mRNA (A). Cells plated onto FN from suspension culture (Ag→FN) did produce low levels of PAI-1 transcripts, whereas plating onto FN in the presence of TGF-β1 (Ag→FN+TGF-β1) yielded optimal induction. PAI-1 induction as a consequence of FN attachment alone was also attenuated by addition of PD98059 during the 2 hour period of adhesion suggesting that MEK activity was also required for adhesion-dependent expression under growth factor-free conditions (B).

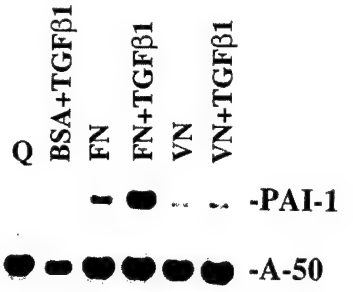

Fig. 8. Matrix-type dependency of basal and TGF-β1-induced PAI-1 expression. Quiescent (Q) T2 cells were trypsinized and replated on plastic dishes coated with BSA, fibronectin (FN) or vitronectin (VN) in the presence or absence of TGF-β1 (1 ng/ml) for a 2 hour period. While both FN and VN induced PAI-1 mRNA transcripts, the amplitude of induction was significantly greater on FN-coated surfaces; TGF-β1 stimulated expression only on T2 cells adhering to FN.

were maintained in non-supplemented medium, stimulated with TGF-β1 (1 ng/ml) in suspension, transferred to fibronectin-coated dishes in serum-/TGF-β1-free medium, or stimulated with TGF-β1 during adhesion to fibronectin (all treatments were for 2 hours). Cells in agarose culture under supplement-free conditions or treated with TGF-β1 in suspension expressed relatively low levels of PAI-1 mRNA compared with the robust expression evident upon stimulation with TGF-β1 during attachment to fibronectin (Fig. 7A). Adhesion to fibronectin matrices alone, in the absence of added TGF-β1, was sufficient to initiate modest PAI-1 transcription (Fig. 7A,B). This adhesion-dependent induction reflected a similarly conservative increase (i.e. threefold) in phospho-ERK1/2 levels (not shown) and, like TGF-β1-mediated expression in normally anchored cells (Fig. 3), also required MEK activity as it was effectively inhibited by PD98059 (Fig. 7B). To further assess if this adhesion-related PAI-1 induction (in either TGF-β1-stimulated or unstimulated cells) was dependent on the nature of the 'matrix' encountered, T2 cells were plated onto dishes coated with 10 μg/ml fibronectin, vitronectin or BSA. TGF-β1 did not induce PAI-1 under non-adherent conditions (i.e., culture on BSA-coated surfaces). Attachment to fibronectin for 4 hours (in the absence of TGF-β1) was an effective inducer of PAI-1 transcripts (by three to fivefold) relative to adhesion to vitronectin (Fig. 8). Moreover, preliminary kinetic determinations indicated that PAI-1 mRNA levels increased as a function of time of attachment suggesting that subsequent cell spreading may be a factor in expression control. This was not unique to T2 cells as a similar response was evident in human dermal fibroblasts and microvessel endothelial cells (data not shown). However, no difference in either the attachment and/or spreading of T2 cells plated on fibronectin- compared with vitronectin-coated surfaces was evident within the same time (Fig. 9). More importantly, TGF-β1 significantly enhanced PAI-1 expression only in T2 cells during attachment to fibronectin (Fig. 8). PAI-1 mRNA levels in cells seeded on vitronectin in the presence of TGF-β1 were not different from that expressed during adhesion to vitronectin alone.

DISCUSSION

Several important aspects of in vivo injury repair (i.e. regional uPA/PAI-1 expression, spatial/temporal distinctions between motile and proliferative phenotypes) (Reidy et al., 1995; Romer et al., 1991; Romer et al., 1994) are recapitulated during cell migration into the denuded areas of a scrape-injured monolayer (Pepper et al., 1987; Pepper et al., 1992; Garlick and Taichman, 1994; Zahm et al., 1997; Providence et al., 2000). PAI-1 is rapidly synthesized by cells immediately adjacent to experimentally-created wounds (Pepper et al., 1992; Pawar et al., 1995; Providence et al., 2000). PAI-1 synthesis and deposition into cellular migration tracks are characteristics of a mobile cohort (Seebacher et al., 1992; Pepper et al., 1992) and an essential component of the migratory program (Providence et al., 2000; Kutz et al., 2001). The in situ distribution of this protein is consistent with a function in cell locomotion. De novo synthesized PAI-1 protein accumulates in the cellular undersurface region, probably in a complex with matrix vitronectin (Seiffert et al., 1994; Lawrence et al., 1997) although it appears that PAI-1 may also associate with fibronectin and/or laminin deposits in migration tracks (Seebacher et al., 1992). Therefore, this SERPIN is well-positioned to modulate integrin-ECM or uPA/uPAR-ECM interactions as well as ECM barrier proteolysis. In vitro studies suggest that PAI-1 may dissociate bound vitronectin from the uPAR, detaching cells that use this receptor as a vitronectin anchor (Deng et al., 1996; Kjoller et al., 1997; Loskutoff et al., 1999). Alternatively, PAI-1 may directly inhibit αv integrin-mediated attachment to vitronectin by blocking accessibility to the RGD sequence located proximal to the uPAR binding site (Stefansson and Lawrence, 1996; Loskutoff et al., 1999), although this inhibition is subject to spatial-temporal constraints (Germer et al., 1998). Furthermore, uPAR-associated uPA/PAI-1 complexes are internalized by endocytosis, which promotes uPA receptor recycling (Andreasen et al., 1997) and thereby

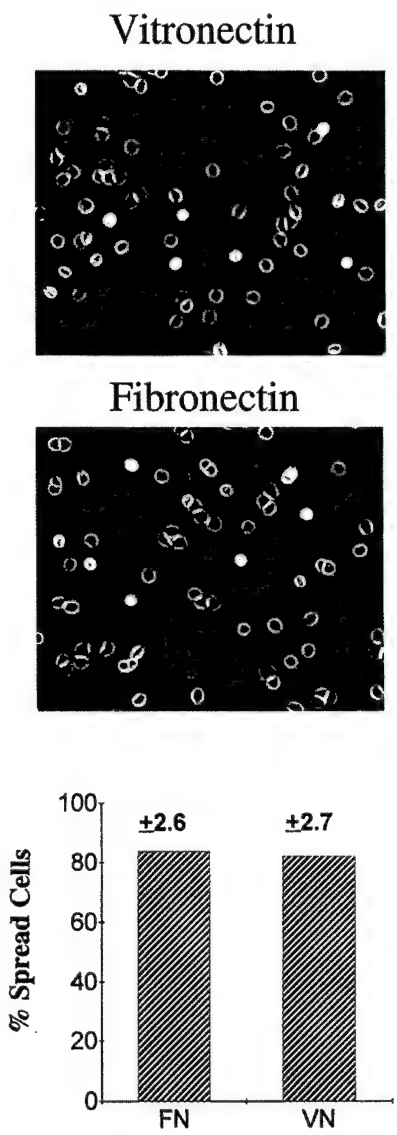

Fig. 9. Relative spreading of T2 cells on fibronectin and vitronectin. Suspended T2 cells were seeded in serum-free medium to dishes previously coated with fibronectin or vitronectin (10 μg/ml). After 4 hours, random fields were photographed (representative examples shown) and the percent spread cells (i.e. non-refractive) calculated. Data plotted are the means±s.d. for assessments on three separate dishes/substrate. There was no difference in either T2 cell attachment or spreading on fibronectin or vitronectin.

vitronectin-dependent cell movement. However, transgenic approaches have suggested that PAI-1 promotes vitronectin-independent angiogenesis specifically by inhibition of plasmin proteolysis, thus preserving an appropriate matrix scaffold or providing required neovessel stability (Bajou et al., 2001). Although in vivo compensatory mechanisms may partly explain this discrepancy between animal and culture models, motility controls clearly vary and depend on the level of expression of participating elements, the nature of the provisional 'matrix' encountered, the context of the system studied and the growth factor environment.

TGF-β1 exerts concentration-dependent effects on cellular locomotion in 3D culture systems (Gajdusek et al., 1993) as well as in the more spatially restricted planar model of denudation injury (this study) (Gajdusek et al., 1993; Zicha et al., 1999). Wound repair analysis of the PAI-1-deficient 4HH cell line, in which PAI-1 synthesis is specifically ablated by antisense targeting (Higgins et al., 1997; Providence et al., 2000), supports the contention that PAI-1 is an important component in the motile program in this model. Since TGF-β1 stimulates PAI-1 synthesis and PAI-1 impacts directly on cell motility (this study) (Deng et al., 1999; Providence et al., 2000), it was important to assess TGF-β1-dependent controls on PAI-1 expression as well as on cellular migration. TGF-β1 initiates PAI-1 transcription in quiescent T2 cells via an immediate-early response, tyrosine kinase-dependent pathway that involves MEK, an upstream activator of ERK1/2. However, unlike the typical rapid ERK phosphorylation associated with serum-stimulation (i.e., within 15 minutes), TGF-β1-mediated ERK activation (as assessed by phosphorylation of the target substrate MBP) was delayed by 30-60 minutes. The MEK dependency for PAI-1 expression and TGF-β1-stimulated as well as basal migration in T2 cells suggests that these events are related. Although MEK blockade probably interferes with cell movement at several levels (Klemke et al., 1997; Rikitake et al., 2000), genetic targeting approaches confirmed that both basal and TGF-β1-stimulated cell migration (over a 24-36 hour period) requires PAI-1 expression (Providence et al., 2000; Kutz et al., 2001). Clearly, MEK inhibition may not affect basal locomotion in all cell types (Nguyen et al., 1998) but results may depend on the specific system studied. For example, in the monolayer denudation model (unlike random motility assays), 'basal' migration is most probably growth factor-mediated. Indeed, monolayer wounding in various cell types, in and of itself, is a sufficient stimulus to initiate autocrine growth factor expression (e.g., TGF-β1, basic FGF, heparin-binding EGF) (Sato and Rifkin, 1988) and activate MAP kinases (Dieckgraefe et al., 1997). Moreover, growth factor synthesis and ERK phosphorylation/nuclear translocation occurs specifically in cells adjacent to the injury site (Dieckgraefe et al., 1997; Song et al., 2000; Ellis et al., 2001) similar to the distribution of locomoting PAI-1-expressing cells (Providence et al., 2000).

Matrix attachment, perhaps as part of the motile response, also stimulates PAI-1 expression. This has particular physiologic relevance. Although the present data suggest that not all matrices have equivalent inductive capability, during the process of wound healing cells 'switch' their integrin complement to accommodate the composition of the provisional ECM (Yamada et al., 1996). In certain instances, TGF-β1 directly mediates changes in integrin availability and, therefore, cellular adhesive traits (Collo and Pepper, 1999; Dalton et al., 1999; Lai et al., 2000). Engagement of particular integrins (i.e. αvβ3, α3β1) by immobilized antibodies or ligands has been implicated in PAI-1 gene control (Ghosh et al., 2000; Khatib et al., 2001). The α3β1 ligands laminin-5 and collagen I, when presented immobilized on beads, were also effective inducers of uPA synthesis (Ghosh et al., 2000). Similar to data reported in this study with regard to adhesive controls on PAI-1 expression, β1 integrin aggregation-induced uPA synthesis was also MEK-dependent as PD98059 inhibited ERK activation and uPA expression. Perhaps not coincidentally, both uPA and PAI-1 are induced by

pharmacologic disorganization of the actin-based microfilament system as is ERK activation (Higgins et al., 1992; Irigoyen et al., 1997). Since integrin ligation/clustering and cell adhesion result in various levels of cytoskeletal reorganization and recruitment of signaling intermediates (Zhu and Assoian, 1995; Lin et al., 1997; Miyamoto et al., 1998; Renshaw et al., 1997), the control of specific protease/protease inhibitors may be a common event in 'outside-in' signaling initiated by adhesive state and integrin engagement. Several matrices (i.e., fibronectin vs vitronectin) clearly differ in relative capacity to induce PAI-1 expression in T2 cells allowed to adhere under growth factor-free conditions. Most novel, however, is the observation that only certain matrix attachments synergize with TGF-β1 to achieve maximal PAI-1 expression. Whether matrix-type variations in the amplitude and duration of ERK signaling underlies this differential response in T2 epithelial cells is currently under study. Nevertheless, the present findings indicate that adhesive influences also modulate TGF-β1 signaling to target genes (i.e. PAI-1).

This work was supported by grants from the NIH (GM57242, GM07033, CA69612) and the Department of the Army (DAMD17-98-1-8015, DAMD17-00-1-0124).

REFERENCES

Akiyoshi, S., Ishii, M., Nemoto, N., Kawabata, M., Aburatani, H. and Miyazano, K. (2001). Targets of transcriptional regulation by transforming growth factor-β: Expression profile analysis using oligonucleotide arrays. *Jpn. J. Cancer Res.* **92**, 252-268.

Andreasen, P. A., Kjoller, L., Christensen, L. and Duffy, M. J. (1997). The urokinase-type plasminogen activator systen in cancer metastasis: a review. *Int. J. Cancer* **72**, 1-22.

Bajou, K., Masson, V., Gerard, R. D., Schmitt, P. M., Albert, V., Praus, M., Lund, L. R., Frandsen, T. L., Brunner, N., Dano, K. et al. (2001). The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications for antiangiogenic stratagies. *J. Cell Biol.* **152**, 777-784.

Boehm, J. R., Kutz, S. M., Sage, E. H., Staiano-Coico, L. and Higgins, P. J. (1999). Growth state-dependent regulation of plasminogen activator inhibitor type-1 gene expression during epithelial cell stimulation by serum and transforming growth factor-β1. *J. Cell. Physiol.* **181**, 96-106.

Boland, S., Boisvieux-Ulrich, E., Houcine, O., Baeza-Squiban, A., Pouchelet, M., Schoevaert, D. and Marano, F. (1996). TGF β1 promotes actin cytoskeleton reorganization and migratory phenotype in epithelial tracheal cells in primary culture. *J. Cell Sci.* **109**, 2207-2219.

Cajot, J. F., Schleuning, W. D., Medcalf, R. L., Bamat, J., Testuz, J., Liebermann, L. and Sordat, B. (1989). Mouse L cells expressing human prourokinase-type plasminogen activator: effects on extracellular matrix degradation and invasion. *J. Cell Biol.* **109**, 915-925.

Cajot, J. F., Bamat, J., Bergonzelli, G. E., Kruithof, E. K., Medcalf, R. L., Testuz, J. and Sordat, B. (1990). Plasminogen-activator inhibitor type 1 is a potent natural inhibitor of extracellular matrix degradation by fibrosarcoma and colon carcinoma cells. *Proc. Natl. Acad. Sci. USA* **87**, 6939-6943.

Chapman, H. A. (1997). Plasminogen activators, integrins, the coordinate regulation of cell adhesion and migration. *Curr. Opin. Cell Biol.* **9**, 714-724.

Ciambrone, G. J. and McKeown-Longo, P. J. (1990). Plasminogen activator inhibitor type 1 stabilizes vitronectin-dependent adhesions in HT-1080 cells. *J. Cell Biol.* **111**, 2183-2195.

Collo, G. and Pepper, M. S. (1999). Endothelial cell integrin α5β1 expression is modulated by cytokines and during migration *in vitro*. *J. Cell Sci.* **112**, 569-578.

Creemers, E., Cleutjens, J., Smits, J., Heymans, S., Moons, L., Collen, D., Daemen, M. and Carmeliet, P. (2000). Disruption of the plasminogen gene in mice abolishes wound healing after myocardial infarction. *Am. J. Pathol.* **156**, 1865-1873.

Dalton, S. L., Scharf, E., Davey, G. and Assoian, R. K. (1999). Transforming growth factor-β overrides the adhesion requirement for surface expression of α5β1 integrin in normal rat kidney fibroblasts. A necessary effect for induction of anchorage-independent growth. *J. Biol. Chem.* **274**, 30139-30145.

Deng, G., Curriden. S. A., Wang, S., Rosenberg, S. and Loskutoff, D. J. (1996). Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? *J. Cell Biol.* **134**, 1563-1571.

Dieckgraefe, B. K., Weems, D. M., Santoro, S. A. and Alpers, D. H. (1997). ERK and p38 MAP kinase pathways are mediators of intestinal epithelial wound-induced signal transduction. *Biochem. Biophys. Res. Comm.* **233**, 389-394.

Ellis, P. D., Hadfield, K. M., Pascall, J. C. and Brown, K. D. (2001). Heparin-binding epidermal-growth-factor-like growth factor gene expression is induced by scrape-wounding epithelial cell monolayers: involvement of mitogen-activated protein kinase cascades. *Biochem. J.* **354**, 99-106.

Fukazawa, H., Uehara, Y., Murakami, Y., Mizuno, S., Hamada, M. and Takeuchi, T. (1994). Labeling of v-Src and BCR-ABL tyrosine kinases with [14C]herbimycin A and its use in the elucidation of the kinase inactivation mechanism. *FEBS Lett.* **340**, 155-158.

Gajdusek, C. M., Luo, Z. and Mayberg, M. R. (1993). Basic fibroblast growth factor and transforming growth factor β-1: synergistic mediators of angiogenesis in vitro. *J. Cell. Physiol.* **157**, 133-144.

Garlick, J. A. and Taichman, L. B. (1994). Fate of human keratinocytes during reepithelialization in an organotypic culture model. *Lab. Invest.* **70**, 916-924.

Germer, M., Kanse, S. M., Kirkegaard, T., Kjoller, L., Felding-habermann, B., Goodman, S. and Preissner, K. T. (1998). Kinetic analysis of integrin-dependent cell adhesion on vitronectin – the inhibitory potential of plasminogen activator inhibitor-1 and RGD peptides. *Eur. J. Biochem.* **253**, 669-674.

Ghosh, S., Brown, R., Jones, J. C. R., Ellerbroek, S. M. and Stack, M. S. (2000). Urinary-type plasminogen activator (uPA) and uPA receptor localization are regulated by α3β1 integrin in oral keratinocytes. *J. Biol. Chem.* **275**, 23869-23876.

Goke, M. N., Cook, J. R., Kunert, K. S., Fini, M. E., Gipson, I. K. and Podolsky, D. K. (2001). Trefoil peptides promote restitution of wounded corneal epitehlial cells. *Exp. Cell Res.* **264**, 337-344.

Greenwood, J. A. and Murphy-Ullrich, J. E. (1998). Signaling of de-adhesion in cellular regulation and motility. *Microsc. Res. Tech.* **43**, 420-432.

Hartsough, M. T. and Mulder, K. M. (1995). Transforming growth factor β activation of p44MAPK in proliferating cultures of epithelial cells. *J. Biol. Chem.* **270**, 7117-7124.

Higgins, P. J., Chaudhari, P. and Ryan, M. P. (1991). Cell-shape regulation and matrix protein p52 content in phenotypic variants of *ras*-transformed rat kidney fibroblasts. *Biochem. J.* **273**, 651-658.

Higgins, P. J., Ryan, M. P. and Ahmed, A. (1992). Cell shape-associated transcriptional activation of the p52(PAI-1) gene in rat kidney cells. *Biochem. J.* **288**, 1017-1024.

Higgins, P. J., Ryan, M. P. and Jelley, D. M. (1997). $p52^{PAI-1}$ gene expression in butyrate-induced flat revertants of v-*ras*-transformed rat kidney cells: mechanism of induction and involvement in the morphologic response. *Biochem. J.* **321**, 431-437.

Irigoyen, J. P., Besser, D. and Nagamine, Y. (1997). Cytoskeleton reorganization induces the urokinase-type plasminogen activator gene via the Ras/extracellular signal-regulated kinase (ERK) signaling pathway. *J. Biol. Chem.* **272**, 1904-1909.

Khatib, A.-M., Nip, J., Fallavollita, L., Lehmann, M., Jensen, G. and Brodt, P. (2001). Regulation of urokinase plasminogen activator/plasmin-mediated invasion of melanoma cells by the integrin vitronectin receptor αvβ3. *Int. J. Cancer* **91**, 300-308.

Kjoller, L., Kanse, S. M., Kirkegaard, T., Rodenburg, K. W., Ronne, E., Goodman, S. L., Preissner, K. T., Ossawski, L. and Andreasen, P. A. (1997). Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. *Exp. Cell Res.* **232**, 420-429.

Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., de Lanerolle, P. and Cheresh, D. A. (1997). Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol.* **137**, 481-492.

Kutz, S. M., Nickey, S., White, L. A. and Higgins, P. J. (1997). Induced PAI-

1 mRNA expression and targeted protein accumulation are early G1 events in serum-stimulated rat kidney cell. *J. Cell. Physiol.* **170**, 8-18.

Kutz, S, M., Providence, K. M. and Higgins, P. J. (2001). Antisense targeting of c-fos transcripts inhibits serum- and TGF-β1-stimulated PAI-1 gene expression and directed motility in renal epithelial cells. *Cell Motil. Cytoskel.* **48**, 163-174.

Lai, C. F., Feng, X., Nishimura, R., Teitelbaum, S. L., Avioli, L. V., Ross, F. P. and Cheng, S. L. (2000). Transforming growth factor-β up-regulates the β5 integrin subunit expression via Sp1 and Smad signaling. *J. Biol. Chem.* **275**, 36400-36406.

Lawrence, D. A., Palaniappan, S., Stefansson, S., Olson, S. T., Francis-Chmura, A. M., Shore, J. D. and Ginsburg, D. (1997). Characterization of the binding of different conformational forms of plasminogen activator inhibitor-1 to vitronectin. Implications for the regulation of pericellular proteolysis. *J. Biol. Chem.* **272**, 7676-7680.

Legrand, C., Polette, M., Tournier, J. M., de Bentzmann, S., Huet, E., Monteau, M. and Birembaut, P. (2001). uPA/plasmin system-mediated MMP-9 activation is implicated in bronchial cell migration. *Exp. Cell Res.* **264**, 326-333.

Lin, T. H., Chen, Q., Howe, A. and Juliano, R. L. (1997). Cell anchorage permits efficient signal transduction between ras and its downstream kinases. *J. Biol. Chem.* **272**, 8849-8852.

Loskutoff, D. J., Curriden, S. A., Hu, G. and Deng, G. (1999). Regulation of cell adhesion by PAI-1. APMIS **107**, 54-61.

Lund, L. R., Romer, J., Bugge, T. H., Nielsen, B. S., Frandsen, T. L., Degen, J. L., Stephens, R. W. and Dano, K. (1999). Functional overlap between two classes of matrix-degrading proteases in wound healing. *EMBO J.* **18**, 4645-4656.

Martin, P. (1997). Wound healing – aiming for perfect skin regeneration. *Science* **276**, 75-81.

Mazzieri, R., Masiero, L., Zanetta, L., Monea, S., Onisto, M., Garbisa, S. and Mignatti, P. (1997). Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulatory mechanism with cell-bound reactants. *EMBO J.* **16**, 2319-2332.

Mignatti, P. and Rifkin, D. B. (2000). Nonenzymatic interactions between proteinases and the cell surface: novel roles in normal and malignant cell physiology. *Adv. Cancer Res.* **78**, 103-157.

Miyamoto, S., Katz, B. Z., Lafrenie, R. and Yamada, K. M. (1998). Fibronectin and integrins in cell adhesion, signaling, and morphogenesis. *Ann. New York Acad. Sci.* **867**, 119-129.

Munz, B., Smola, H., Engelhardt, F., Bleuel, K., Brauchle, M., Lein, I., Evans, L. W., Huylebroeck, D., Balling, R. and Werner, S. (1999). Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. *EMBO J.* **18**, 5205-5215.

Nguyen, D. H. D., Hussaini, I. M. and Gonias, S. L. (1998). Binding of urokinase-type plasminogen activator to its receptor in MCF-7 cells activates extracellular signal-regulated kinase 1 and 2 which is required for increased cellular motility. *J. Biol. Chem.* **273**, 8502-8507.

Okedon, L. E., Sato, Y. and Rifkin, D. B. (1992). Urokinase-type plasminogen activator mediates basic growth factor-induced bovine endothelial migration independent of its proteolytic activity. *J. Cell. Physiol.* **150**, 258-263.

Pawar, S., Kartha, S. and Toback, F. G. (1995). Differential gene expression in migrating renal epithelial cells after wounding. *J. Cell. Physiol.* **165**, 556-565.

Pepper, M. S., Vassalli, J. D., Montesano, R. and Orci, L. (1987). Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. *J. Cell Biol.* **105**, 2535-2541.

Pepper, M. S., Sappino, A. P., Montesano, R., Orci, L. and Vassalli, J. D. (1992). Plasminogen activator inhibitor-1 is induced in migrating capillary endothelial cells. *J. Cell. Physiol.* **153**, 129-139.

Pilcher, B. K., Dumin, J., Schwartz, M. J., Mast, B. A., Schultz, G. S., Parks, W. C. and Welgus, H. G. (1999). Keratinocyte collagenase-1 expression requires an epidermal growth factor receptor autocrine mechanism. *J. Biol. Chem.* **274**, 10372-10381.

Providence, K. M., Kutz, S. M., Staiano-Coico, L. and Higgins, P. J. (2000). PAI-1 gene expression is regionally induced in wounded epithelial cell monolayers and required for injury repair. *J. Cell. Physiol.* **182**, 269-280.

Reidy, M., Irwin, C. and Linder, V. (1995). Migration of arterial wall cells. *Circ. Res.* **78**, 405-414.

Renshaw, M. W., Ren, X-D. and Schwartz, M. A. (1997). Growth factor activation of MAP kinase requires cell adhesion. *EMBO J.* **16**, 5592-5599.

Rheinwald, J. G., Jorgensen, J. L., Hahn, W. C., Terpstra, A. J., O'Connell, T. M. and Plummer, K. K. (1987). Mesosecrin: a secreted glycoprotein produced in abundance by human mesothelial, endothelial, and kidney epithelial cells in culture. *J. Cell Sci.* **104**, 263-275.

Rikitake, Y., Kawashima, S., Yamashita, T., Ueyama, T., Ishido, S., Hotta, H., Hirata, K-I. and Yokoyama, M. (2000). Lysophosphatidylcholine inhibits endothelial cell migration and proliferation via inhibition of the extracellular signal-regulated kinase pathway. *Arterioscler. Thromb. Vasc. Biol.* **20**, 1006-1012.

Romer, J., Lund, L. R., Kriksen, J., Ralkiaer, E., Zeheb, R., Gelehrter, T. D., Dano, K. and Kristensen, P. (1991). Differential expression of urokinase-type plasminogen activator and its type-1 inhibitor during healing of mouse skin wounds. *J. Invest. Dermatol.* **97**, 803-811.

Romer, J., Lund, L. R., Kriksen, J., Pyke, C., Kristensen, P. and Dano, K. (1994). The receptor for urokinase-type plasmingoen activator is expressed by keratinocytes at the leading edge during re-epithelialization of mouse skin wounds. *J. Invest. Dermatol.* **102**, 519-522.

Romer, J., Bugge, T. H., Pyke, C., Lund, L. R., Flick, M. J., Degen, J. L. and Dano, K. (1996). Impaired wound healing in mice with a disrupted plasminogen gene. *Nat. Med.* **2**, 287-292.

Roovers, K., Davey, G., Zhu, X., Bottazzi, M. E. and Assoian, R. K. (1999). α5β1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. *Mol. Biol. Cell.* **10**, 3197-3204.

Ryan, M. P. and Higgins, P. J. (1993). Growth state-regulated expression of p52(PAI-1) in normal rat kidney cells. *J. Cell. Physiol.* **155**, 376-384.

Ryan, M. P., Kutz, S. M. and Higgins, P. J. (1996). Complex regulation of plasminogen activator inhibitor type-1 (PAI-1) gene expression by serum and substrate adhesion. *Biochem. J.* **314**, 1041-1046.

Sato, Y. and Rifkin, D. B. (1988). Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen ativator synthesis, and DNA synthesis. *J. Cell Biol.* **107**, 1199-1205.

Seebacher, T., Manske, M., Zoller, J., Crabb, J. and Bade, E. G. (1992). The EGF-inducible protein EIP-1 of migrating normal and malignant rat liver epithelial cells is identical to plasminogen activator inhibitor 1 and is a component of the migration tracks. *Exp. Cell Res.* **203**, 504-507.

Seiffert, D., Mimuro, J., Schleef, R. R. and Loskutoff, D. J. (1994). Interactions between type 1 plasminogen activator inhibitor, extracellular matrix and vitronectin. *Cell Differ. Dev.* **32**, 287-292.

Song, Q. H., Singh, R. P., Richardson, T. P., Nugent, M. A. and Trinkaus-Randall, V. (2000). Transforming growth factor-β1 expression in cultured corneal fibroblasts in response to injury. *J. Cell. Biochem.* **77**, 186-199.

Stefansson, S. and Lawrence, D. A. (1996). The serpin PAI-1 inhibits cell migration by blocking αvβ5 binding to vitronectin. *Nature* **383**, 441-443.

Wrana, J. L., Overall, C. M. and Sodek, J. (1991). Regulation of the expression of a secreted acidic protein rich is cysteine (SPARC) in human fibroblasts by transforming growth factor β. Comparison of transcriptional and post-transcriptional control with fibronectin and type I collagen. *Eur. J. Biochem.* **197**, 519-528.

Wysocki, A. B., Kusakabe, A. O., Chang, S. and Tuan T. L. (1999). Temporal expression of urokinase plasminogen activator, plasminogen activator inhibitor and gelatinase-B in chronic wound fluid switches from a chronic to acute wound profile with progression to healing. *Wound Repair Regen.* **7**, 154-165.

Xie, H., Pallero, M. A., Gupta, K., Chang, P., Ware, M. F., Witke, W., Kwiatkowski, D. J., Lauffenburger, D. A., Murphy-Ullrich, J. E. and Wells, A. (1998). EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLCγ signaling pathway. *J. Cell Sci.* **111**, 615-624.

Yamada, K. M. and Clark, R. A. F. (1996). Provisional matrix. In *The Molecular Biology of Wound Repair* (ed. R. A. F. Clark), pp. 51-93. Plenum Press, NY.

Yamada, K. M., Gailit, J. and Clark, R. A. F. (1996). Integrins in wound repair. In *The Molecular and Cellular Biology of Wound Repair* (ed. R. A. F. Clark), pp. 311-354. Plenum Press, NY.

Yonekura, A., Osaki, M., Hirota, Y., Tsukazaki, T., Miyazaki, Y., Matsumoto, T., Ohtsuru, A., Namba, H., Shindo, H. and Yamashita, S. (1999). Transforming growth factor-β stimulates articular chondrocyte cell growth through p44/42 MAP kinase (ERK) activation. *Endocr. J.* **46**, 545-553.

Zahm, J. M., Kaplan, H., Herar, A. L., Doriot, F., Pierrot, D., Somelette, P. and Puchelle, E. (1997). Cell migration and proliferation during the in vitro wound repair of the respiratory epithelium. *Cell Motil. Cytoskel.* **37**, 33-43.

Zambruno, G., Marchisio, P. C., Marconi, A., Vaschieri, C., Melchiori, A.,

Giannetti, A. and De Luca, M. (1995). Transforming growth factor-β1 modulates β1 and β5 integrin receptors and induces the *de novo* expression of the αvβ6 heterodimer in normal human keratinocytes: implications for wound healing. *J. Cell Biol.* **129**, 853-865.

Zavadil, J., Bitzer, M., Liang, D., Yang, Y-C., Massimi, A., Kneitz, S., Piek, E. and Bottinger, E. P. (2001). Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. *Proc. Natl. Acad. Sci. USA* **98**, 6686-6691.

Zhou, H. M., Nichols, A., Meda, P. and Vassalli, J. D. (2000). Urokinase-type plasminogen activator and its receptor synergize to promote pathogenic proteolysis. *EMBO J.* **19**, 4817-4826.

Zhu, X. and Assoian, R. K. (1995). Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. *Mol. Biol. Cell* **6**, 273-282.

Zicha, D., Genot, E., Dunn, G. A. and Kramer, I. M. (1999). TGFβ1 induces a cell-cycle-dependent increase in motility of epithelial cells. *J. Cell Sci.* **112**, 447-454.

Cell Motility and the Cytoskeleton 48:163–174 (2001)

Antisense Targeting of c-*fos* Transcripts Inhibits Serum- and TGF-β1-Stimulated PAI-1 Gene Expression and Directed Motility in Renal Epithelial Cells

Stacie M. Kutz, Kirwin M. Providence, and Paul J. Higgins*

Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York

Plasminogen activator inhibitor type-1 (PAI-1), the major regulator of pericellular plasmin generation, and the c-FOS transcription factor are expressed by migrating cells in response to monolayer wounding. Induced c-*fos* and PAI-1 transcripts were evident within 30 min and 2 h, respectively, of scrape injury to confluent, growth-arrested, cultures of NRK epithelial cells. Since c-FOS/AP-1 DNA-binding activity modulates both basal and inducible modes of PAI-1 gene control, and AP-1 motif binding factors were present in quiescent as well as stimulated NRK cells, a model of directionally regulated cell movement (migration into scrape-denuded "wounds") was used to assess the consequences of c-*fos* transcript targeting on PAI-1 expression and cell motility. This in vitro model of epithelial injury closely approximated in vivo wound repair with regard to the spatial and temporal emergence of cohorts of cells involved in migration, proliferation, and PAI-1 expression. Stable cell lines (NRK*sof*) were generated by transfection of parental NRK cells with a c-*fos* antisense expression vector. Serum-inducible c-*fos* transcripts and PAI-1 protein levels were significantly attenuated in NRK*sof* transfectants relative to parental controls or cells transfected with a neo^R vector without the *sof* insert. NRK*sof* cells had a markedly impaired ability to repair scrape-generated monolayer wounds under basal, serum-stimulated, or TGF-β1-supplemented culture conditions. Since injury closure and PAI-1 induction were attenuated in c-*fos* antisense cells, it was important to clarify the role of specific AP-1 sites in serum-mediated PAI-1 transcription. PAI-1 "promoter"-driven CAT reporter expression was assessed within the real time of serum-stimulated PAI-1 induction. A segment of the PAI-1 promoter corresponding to nucleotides −533 to −764 upstream of the transcription start site functioned as a prominent serum-responsive region (SSR). The 9-fold increase in CAT mRNA levels attained with the −533 to −764 bp PAI-1 SRR ligated to a minimal PAI-1 promoter (i.e., 162 bp of 5′ flanking sequence containing the basal transcription complex) closely approximated the serum-induced transcriptional activity of a fully responsive (1,230 bp) PAI-1 promoter construct as well as the endogenous PAI-1 gene. AP-1-like, CTF/NF-1-like, and AP-2 sites were identified in the SRR. The SRR AP-1 motif was homologous to the sequence TGACACA that mapped between nucleotides −740 and −703 in the human PAI-1 gene, a region essential for growth factor-inducible PAI-1 transcription. While the functionality of this AP-1 site in wound-regulated PAI-1 synthesis remains to be determined, antisense c-*fos*

Contract grant sponsor: National Institutes of Health; Contract grant numbers: DK46272, GM42461; Contract grant sponsor: Department of the Army; Contract grant number: DAMD 17-98-1-8015.

*Correspondence: Dr. Paul J. Higgins, Center for Cell Biology and Cancer Research, Albany Medical College (Mail Code 165), 47 New Scotland Avenue, Albany, NY 12208.
E-mail: higginp@mail.amc.edu

Received 18 April 2000; Accepted 27 September 2000

transcripts effectively attenuated PAI-1 induction and basal as well as growth factor-stimulated cell locomotion, indicating that expression of both the PAI-1 and c-*fos* genes is necessary for wound-initiated NRK cell migration. Cell Motil. Cytoskeleton 48:163–174, 2001.

Key words: protease inhibition; wound repair; transcription; c-*fos*

INTRODUCTION

Mitogenic stimulation of quiescent (G_o) cells initiates a temporally regulated program of genomic activity with induced genes segregating among the immediate-early, delayed-early, and late response sets [Lau and Nathans, 1987; Almendral et al., 1988; Lanahan et al., 1992; Muller et al., 1993; Vincent et al., 1993; Burger et al., 1994]. Immediate-early response (IER) genes are expressed either transiently during the $G_o \rightarrow G_1$ period (e.g., c-*fos*) or throughout G_1 phase (e.g., c-*myc*) [Lau and Nathans, 1987; Kato and Dang, 1992]. IER genes are also activated following tissue injury with inductive kinetics generally similar to that of serum-stimulated cells [Pawar et al., 1995; Ryan et al., 1996; Providence et al., 2000]. One subset of IER genes encodes proteins that structurally link the extracellular matrix (ECM) and the internal cytoskeleton (e.g., actin, α-tropomyosin, fibronectin, vinculin, α-actinin, β_1-integrin) [Ryseck et al., 1989; Muller et al., 1993]. Expression of this complement of IER genes likely reflects the specific cytoarchitectural and adhesive requirements for wound-induced cell proliferation and motility [Folkman and Moscona, 1978; Guadagno et al., 1993; Lauffenburger and Horwitz, 1996; Greenwood and Murphy-Ullrich, 1998]. A second IER gene group encodes matrix-active proteases and their inhibitors (e.g., urokinase plasminogen activator [uPA] and its type-1 inhibitor [PAI-1]) [Grimaldi et al., 1986; Ryan et al., 1996]. PAI-1 maintains the structure and integrity of the ECM by suppressing uPA-dependent plasmin generation [e.g., Pollanen et al., 1991]. PAI-1 also influences cellular migratory activity by modulating interactions between vitronectin, and either the uPA receptor (uPAR) or αv integrins [Stefansson and Lawrence, 1996; Kanse et al., 1996; Waltz et al., 1997; Kjoller et al., 1997; Loskutoff et al., 1999]. Mediation of cell attachment and spreading by PAI-1 likely requires uPA and appears partly dependent on fibronectin [Planus et al., 1997; Liu et al., 1999]. The dynamics of uPA and PAI-1 synthesis/localization, therefore, may dictate the formation or longevity of cell-to-matrix contact structures required for growth or migration [Mignatti and Rifkin, 2000]. Focalized cell surface uPA and matrix-bound PAI-1, moreover, are well positioned to regulate movement by direct alteration of integrin-ECM adhesions [Deng et al., 1996; Stefansson and Lawrence, 1996; Blasi, 1996; Chapman, 1997]. As cycles of leading edge adhesion/trailing edge detachment must be maintained for cells to locomote effectively [Greenwood and Murphy-Ullrich, 1998], targeting of de novo–synthesized PAI-1 to the cellular undersurface in close proximity to focal contact sites [Kutz et al., 1997] provides an opportunity to influence both uPA-dependent proteolysis and cell attachment [Okedon et al., 1992; Loskutoff et al., 1999].

PAI-1 transcription occurs transiently and in IER fashion upon addition of serum to quiescent cells [Ryan et al., 1996; Boehm et al., 1999]. Similar kinetics of PAI-1 gene control accompany compensatory regeneration subsequent to tissue injury in vivo [Schneiderman et al., 1993; Thornton et al., 1994] or in vitro [Pawar et al., 1995; Providence et al., 2000]. In such circumstances, induction is transcriptional and maximal between 3–8 h post-stimulation with a return to basal levels thereafter [Ryan et al., 1996; Thornton et al., 1994; Providence et al., 2000]. Certain IER genes (including PAI-1) utilize AP-1 sequences, and preexisting AP-1-binding proteins (e.g., c-FOS, c-JUN), to initiate transcription during reentry of growth-arrested cells into the proliferative cycle [Almendral et al., 1988; Arts et al., 1996]. Wounding is a sufficient stimulus to induce transcription of both the c-*fos* and PAI-1 genes as well as recruit quiescent epithelial cells into DNA synthetic phase [Verrier et al., 1986; Pawar et al., 1995; Providence et al., 2000]. While PAI-1 is an important component in the motile apparatus of at least some cell types [reviewed in Mignatti and Rifkin, 2000; Providence et al., 2000], the contribution of c-*fos* to either cell migration and/or PAI-1 induction is unknown. Since the available data suggest that cell cycle phase-specific constraints are superimposed on growth state-dependent transcriptional regulation of the PAI-1 gene [e.g., Boehm et al., 1999], we have utilized the normal rat kidney (NRK) epithelial model system [Kutz et al., 1997] to study molecular events associated with PAI-1 gene expression within the context of renal cell growth activation and its relevance to the process of directed cell motility.

MATERIALS AND METHODS

Cell Culture

Renal epithelial (NRK-52E) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supple-

mented with 10% (v/v) fetal bovine serum (FBS). Three day post-confluent cultures (maintained as described in the text) were stimulated by addition of fresh FBS (20% final concentration)-containing DMEM or by scrape-wounding with a pipette tip. The time course of injury closure was quantified by micrometer measurements of 20 randomly selected wound fields. To identify DNA-synthesizing cells, 5-bromo-2′-deoxyuridine (BrdU; 200 μmol/l final concentration) was added at the time of wounding. The number and location of cells that incorporated the analogue (S-phase cells) were quantified by immunofluorescence microscopy using antibodies to BrdU. Cellular uPAR levels were assessed by Western blotting. Transforming growth factor-β1 (TGF-β1; 1 ng/ml) served as a positive control for c-*fos* and PAI-1 induction in NRK cells [Boehm et al., 1999].

Metabolic Labeling And Gel Electrophoresis

Hanks' balanced salt solution (HBSS)-washed cells were labeled with ^{35}S-methionine (specific activity = 1,100 Ci/mmol; 50 μCi/ml) at 37°C for 6 h. The conditioned labeling medium (containing secreted proteins [SP]) was clarified by centrifugation at 13,000*g* for 5 min. Monolayers were washed twice with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (CMF-PBS) and extracted with saponin (0.2% [w/v] in CMF-PBS) while rotating gently on an orbit shaker at room temperature; cells were subsequently dislodged with a stream of CMF-PBS. The substrate-attached saponin-resistant (SAP fraction) material was solubilized in sample buffer (50 mM Tris-HCl, pH 6.8, 10% [v/v] glycerol, 1% [w/v] sodium dodecyl sulfate [SDS], 1% [v/v] 2-mercaptoethanol). SP and SAP fractions were further diluted in sample buffer, boiled for 3 min and aliquots equivalent to 25,000 cpm trichloroacetic acid-insoluble protein separated on SDS-10% acrylamide slab gels. Radiolabeled proteins were visualized by fluorography of En^{3}Hance- permeabilized gels. PAI-1 immune-precipitated from the metabolically-labeled SP protein complement of 20% dialyzed FBS-stimulated NRK cells [Higgins et al., 1990] provided a PAI-1 position "marker" for electrophoretic separations.

Northern Blot Analysis

Cellular RNA was electrophoretically separated, transferred to Nytran membranes using 10× SSPE (1.8 M NaCl, 100 mM $Na_2HPO_4{\cdot}H_2O$, pH 7.4, 10 mM ethylenediaminetetraacetic acid [EDTA]) and UV crosslinked. Blots were prehybridized at 42°C for 2 h in 50% formamide, 5× Denhardt's reagent, 1% SDS, 100 μg/ml salmon sperm DNA, 5× SSPE buffer then hybridized at 42°C for 16 h with 5×10^6 cpm ^{32}P-dCTP-labeled cDNA probes to PAI-1, actin, c-*fos*, GAPD or A-50 in 50% formamide, 2.5× Denhardt's reagent, 1% SDS, 100 μg/ml salmon sperm DNA, 5× SSPE, and 10% dextran sulfate. Membranes were washed 3 times (15 min each) at 42°C in 0.1% SSPE/0.1% SDS followed by 3 washes at 55°C and exposed to X-OMAT AR-5 film. Linearized pTRI-c-*fos* (containing mouse c-*fos* exon 2) was used to generate T7 RNA polymerase-directed ^{32}P-labeled antisense riboprobes. Prehybridization/hybridization for riboprobes were done at 60°C using buffers described above; blots were washed 3 times in 1× SSPE/0.5% SDS for 15 min each at 65°C followed by one 15-min wash in 1× SSPE/0.1% SDS at 60°C prior to autoradiography.

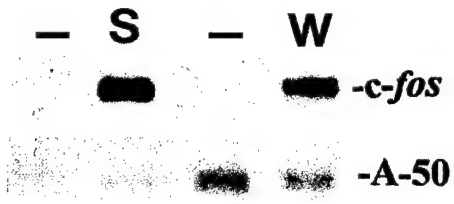

Fig. 1. Relative abundance of c-*fos* transcripts induced in serum- and wound-stimulated NRK cells. RNA was isolated from confluency-arrested quiescent NRK cells (—) as well as from serum-stimulated (S) or multiple scrape-wounded (W) cultures. Total exposure to serum or time post-injury was 30 min prior to RNA harvest. Northern blots were hybridized with ^{32}P-labeled probes to c-*fos* and A-50.

Disruption of c-FOS Expression

To create the FOS antisense expression vector SV-*neo-sof* (gift of Dr. Eileen Adamson), the BglII/HindIII region of the SV40 T antigen gene in pKSV10 was replaced with a 1.7 kb BglII/HindIII fragment of the murine c-*fos* genomic sequence (containing the first exon and part of the second exon) cloned in antisense orientation downstream of an SV40 early promoter. *Neo* coding sequences (1.1-kb) from p*Neo* was inserted into the BglII site. The 3.8-kb transcript expressed from this construct includes *neo* coding and c-*fos* antisense sequences [Edwards et al., 1988]. pSV*neo-sof* or control *neo* plasmids were transfected into NRK cells and populations expanded in G418 (300 μg/ml).

Nuclear Extracts

Cells were suspended in 400 μl of cold 10 mM HEPES, pH 7.9, buffer containing 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, swelled on ice for 15 minutes, 25 μl of 10% NP-40 added and vortexed for 10 sec. Nuclei were collected by centrifugation, resuspended in 50 μl of cold lysis buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1mM EGTA, 1mM DTT) containing leupeptin, aprotinin, chymostatin, pepstatin A, and antipain (each at a final concentration of 10 μg/ml) and rocked at 4°C for 15 min. Nuclear extracts were clarified by centrifugation.

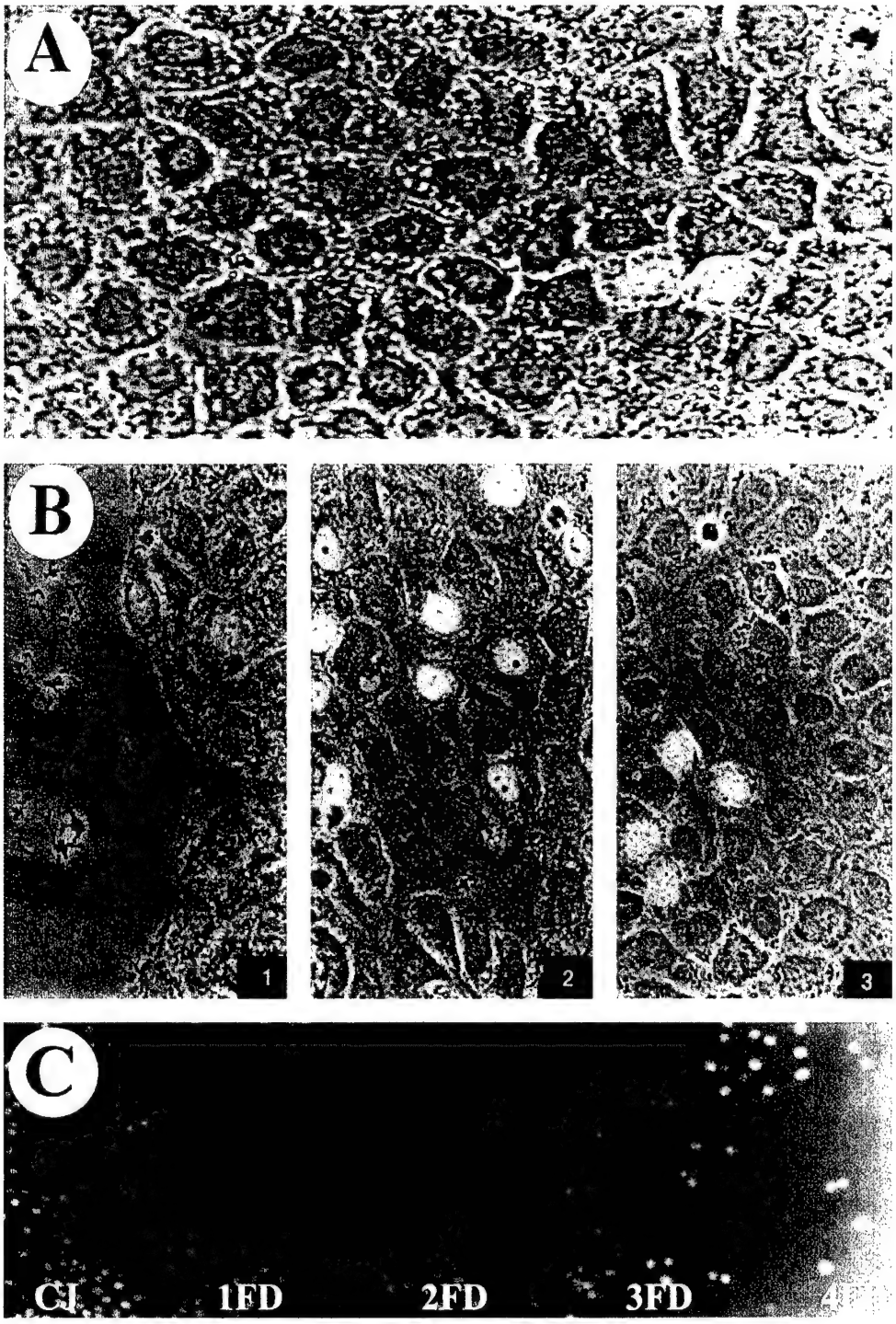

Figure 2.

Reporter Plasmids

Various contiguous regions (from nucleotides −1230, −764, and −266 to +23) of PAI-1 5′genomic flanking sequence were cloned into the chloramphenicol acetyltransferase expression plasmid pCAT(An) creating the constructs rPAI-CAT(-1230), rPAI-CAT(-764), and rPAI-CAT(-266), respectively [Johnson et al., 1992]. rPAI-CAT(-528) and rPAI-CAT(-162) were derived by digesting rPAI-CAT(-1230) with AflI and SalI or Bbr PI and SalI, respectively. rPAI-CAT(-764) was restricted

Fig. 2. Region-specific entry of wounded NRK cell monolayer into DNA synthetic phase. Cells were grown to confluency in DMEM/10% FBS, then maintained in this medium to initiate a condition of growth-arrest. In control intact monolayers, there are relatively few S phase cells (2.9% of the total population as determined by BrdU Incorporation) (**A**). Entry of cells into S phase after wounding is a function of both time and spatial relationship to the injury site. By 12 h post-trauma, leading edge cells have a labeling index approximately the same (3.9%) as an unwounded monolayer (**B1**) increasing to 22.4% at 24 h. Increases in the fraction of S phase cells in a region 1–2 mm from the wound were moderate (6.0%) at 12 h (**B2**) and highly significant (39.3%) by 24 hr. At all times, however, labeling indices in the monolayer distal (40 mm) from the site of injury (**B3**) were similar to that of growth-arrested control cultures (2.2 and 2.9% at 12 and 24 h post-scraping). Upon wound closure, the leading edge cells at the closure junction (CJ) enter S phase in a synchronous manner (90% BrdU-positive junction region cells) approximately 42 h after initial injury (**C**). At this time, and with increasing distance from the closure site (1, 2, 3, or 4 field diameters; FD), there is a marked decrease in the fraction of S phase cells (70.1 and 8.8% at 1–2 and 40 mm, respectively, from the original wound edge). Graphic representation of the time- and location-dependent increase in DNA synthesis in wounded monolayers (**D**). Percent S phase (BrdU-immunoreactive) cells was assessed by visual examination of 10 random fields representative of the proximal (1–2 mm from the injury site) and distal (40 mm from the scrape border) regions of wounded cultures. Data in D = the mean value of 3 independent experiments.

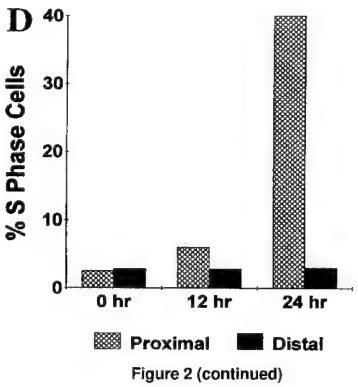

Figure 2 (continued)

with BfrI and BbrPI to delete nucleotides −162 to −533 generating p764-CAT(del 162-533). Purified plasmid fragments were digested in 0.03 M sodium acetate, pH 4.6, 0.05 M NaCl, 1 mM $ZnCl_2$, 5% glycerol, 32 U mung bean nuclease buffer for 1 h at 37°C. DNA was extracted with phenol:chloroform, precipitated with ethanol, washed once with 70% ethanol, and resuspended in TE buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA). Digested plasmids were ligated in buffer containing 50 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 1 mM DTT, 0.25 mM rATP and 6 U T4 DNA ligase at room temperature overnight and transformed into *Escherichia coli* HB101.

Transfection and Assessment of CAT mRNA Abundance

Cells (in 100-mm dishes) were washed in antibiotic- and serum-free DMEM; lipofectAMINE (28 μl/dish)-DNA (4 μg/dish) complexes were added and cultures incubated at 37°C for 5 h prior to addition of DMEM/10% FBS. The following day, the cells were washed with CMF-HBSS, trypsinized and divided into 2 dishes containing DMEM/10% FBS; 24 h later, the medium was removed, cells washed twice with CMF-HBSS, and serum-free DMEM added to initiate a state of quiescence over the subsequent 3 days. Cells were either maintained under quiescent conditions or stimulated by addition of serum (20% final concentration), then trypsinized 4 h later, distributed into 2 microfuge tubes, collected at 10,000*g*, and washed with CMF-HBSS. One tube was used for extraction of RNA (for assessment of CAT mRNA abundance by hybridization with a CAT-specific cDNA probe [Providence et al., 1999]) while the other was HIRT-extracted (for calculation of transfection efficiency). Southern analysis of HIRT-extracted plasmid DNA was used to normalize CAT mRNA expression values [Providence et al., 1999].

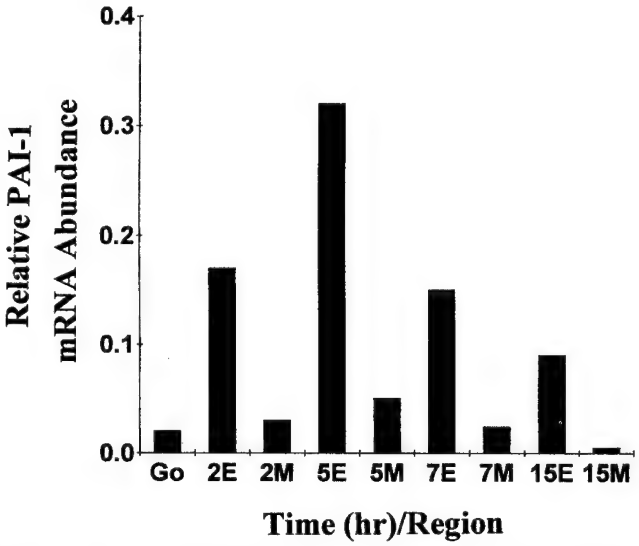

Fig. 3. Changes in PAI-1 mRNA abundance in NRK cell cultures as a function of time after wounding and distance from the injury site. Induction of PAI-1 expression in edge isolate (E) cells is evident within 2 h post-scrape injury, increases to a maximum at approximately 5 h and declines rapidly thereafter. In contrast, the level of PAI-1 transcripts in the distal unwounded monolayer (M) region remains relatively unchanged over the same time course. Data represents the average PAI-1/A-50 values for densitometric analysis of Northern blots of two independent experiments.

Mobility Shift Assay

The PAI-1 5′ flanking genomic sequence from −533 to −764 bp was body-labeled by PCR. A mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.9 mM $MgCl_2$, 0.05 mM dGTP, dTTP and dATP, 100 ng rPAI-CAT(-1230), 10 pmol of each primer (5′-dAGCTTTCTGTGGTAACCC-3′ [nt −764 to −747] and 5′-dGAAGTTCTCTATTGGGCTT-3′ [nt −549 to −533]), 50 μCi ^{32}P-dCTP (3,000 Ci/mmol) and

Taq polymerase (5 U) was prepared in a final volume of 50 μl. Unlabeled PCR products were derived as above using 0.05 mM dATP, dCTP, dGTP, dTTP, and 50 pmol of each primer in the reaction mixture. A double-stranded deoxyoligonucleotide (3–5 pM) representing the AP-1 consensus sequence (5′-dTTCCGGC**TGACTCA**TCA-AGCG-3′) was incubated at 37°C for 10 min with T4 polynucleotide kinase (5–10 U/μl) in 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM $MgCl_2$, 5 mM DTT and ^{32}P-dATP (3,000 Ci/mmol). Probe was purified by filtration through 10,000 mw cellulose spin columns. Nuclear proteins (10–20 μg) were diluted in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl) containing 2 μg poly dI · dC in a final volume of 20 μl. Protein/DNA complexes were allowed to form by the addition of 30,000–60,000 cpm labeled probe followed by incubation at room temperature for 20 min (competition experiments included 100-fold excess unlabeled construct). Following room temperature incubation for 20 min, gel loading buffer (25 mM Tris-HCl, pH 7.5, 0.02% bromophenol blue, 0.02% xylene cyanol, 4% glycerol) was added. Complexes were separated on Tris/glycine gels (Tris/glycine buffer [50 mM Tris HC1, 2 mM EDTA, 100 mM glycine] containing 4% polyacrylamide, 0.05% bis-acrylamide, 2.5% glycerol, 0.075% ammonium persulfate, 0.085% TEMED) for 2 h at 100 V. Gels were exposed to X-OMAT AR-5 film for 24–72 h.

RESULTS

Confluent, growth-arrested, NRK cells expressed c-*fos* mRNA within 30 min after scrape injury (Fig. 1). Quantitative assessments of Northern blots normalized to A-50 mRNA signal suggested that the amplitude of wound-induced c-*fos* transcripts was ≤25% that of serum-activated cultures. Since c-*fos* expression even in multiple scrape-injured monolayers is restricted to cells immediately adjacent to the wound site [Verrier et al., 1986], unlike the global stimulation following fresh serum addition, analysis of entire culture populations likely underestimates c-*fos* mRNA levels in cells specifically involved in the repair process (i.e., the locomoting cohort that comprise the migrating front). The relatively rapid induction of c-*fos* expression, moreover, was one aspect in a regionally activated program of wound resolution involving both temporally-dependent and site-specific changes in DNA synthesis, uPAR expression, and PAI-1 mRNA abundance. Wounding alone was a sufficient stimulus for eventual re-entry of quiescent NRK cells into the cell cycle (Fig. 2) suggesting that, like exposure to mitogenic growth factors [e.g., Almendral et al., 1988], injury-associated c-*fos* induction is part of the proliferative response. Migration into the denuded zone, however, did not involve recruitment of cells at the leading edge into S phase, at least in the initial phase of trauma repair (Fig. 2A,B). A marked increase in S phase cells (from 6% at 12 h post-scraping to 40% at 24 h) was confined to a region 1 to 2 mm parallel to the long axis of the wound whereas the labeling index in the distal monolayer did not exceed 3% (Fig. 2D). Approximately 8 to 12 h after injury closure, but not before, cells within the newly healed wound bed entered S phase in approximate synchronous fashion (Fig. 2B,C). These results are consistent with previous findings that transient mitotic activity restricted to a zone behind the migrating front is necessary to sustain the reepithelization process [Bereiter-Hahn,1984]. The physiologic relevance of this simple in vitro model is further supported by the regional compartmentalization of both uPAR and PAI-1 expression as a function of time post-trauma. Harvesting of cells bordering the injury site, as well as within the distal uninvolved monolayer region, indicated that increases in PAI-1 expression (Fig. 3) and uPAR levels (Fig. 4), like that of c-*fos* [Verrier et al., 1986], was restricted to cells proximal to the wound and involved in the repair process. PAI-1 transcripts were induced early after monolayer scraping, increased over 2 to 5 h post-injury, and declined thereafter (Fig. 3). It appears, therefore, that distinct functional cohorts of renal epithelial cells are induced in response to monolayer trauma as a consequence of time post-injury and relative distance from the wound site.

Addition of fresh serum-containing medium or TGF-β1 to contact-inhibited quiescent NRK cells induced both c-*fos* and PAI-1 transcripts (Fig. 5) within 30 min and 2 h, respectively, of exposure and stimulated the rate of wound repair (Table I) without influencing the incidence of S phase cells in the migratory front. There was, in fact, a marked similarity between the kinetics of PAI-1 expression in wounded (Fig. 3) and serum-stimulated (Fig. 5) cultures; in both cases, PAI-1 induction was preceded by rapid and transient increases in c-*fos* mRNA (Figs. 1 and 5). Serum growth factors induce not only c-*fos* expression but recruit c-FOS into AP-1 DNA-binding complexes with modulation of AP-1-dependent transcription [Felts et al., 1997; Jin and Howe, 1999]. PAI-1 gene activation in response to serum stimulation, moreover, has immediate-early metabolic characteristics [Ryan et al., 1996] further suggesting that such motifs are not only important but likely utilize pre-existing transcriptional control elements. Indeed, AP-1 sites, and c-FOS and c-JUN in particular, function in both the basal and inducible phases of PAI-1 gene control [Keeton et al., 1991; Descheemaeker et al., 1992; Arts et al., 1996]. Nuclear proteins from quiescent as well as serum-stimulated NRK cells, in fact, bound AP-1 consensus sequence deoxyoligonucleotide probes (Fig. 6) and c-FOS

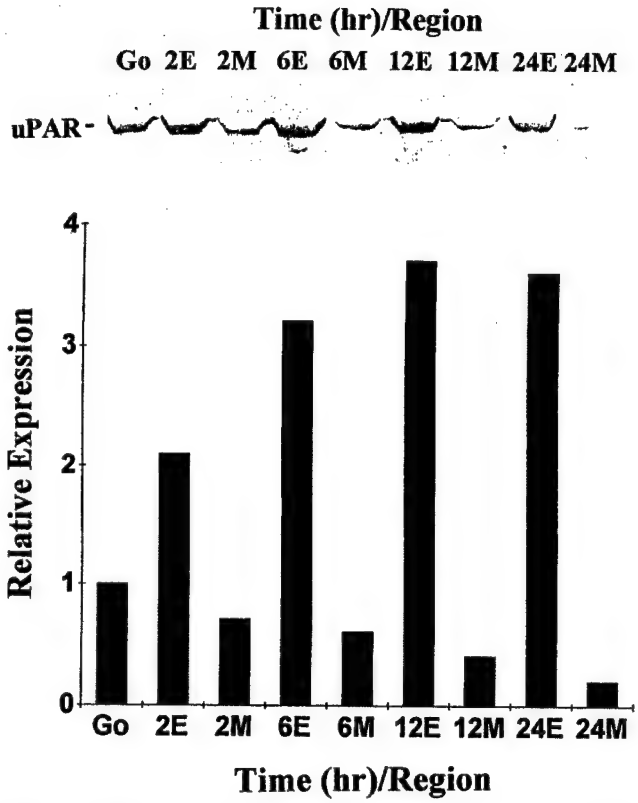

Fig. 4. Western blot analysis of relative uPAR expression by NRK cells as a function of time after monolayer scrape wounding and distance from the injury site. Extracts containing 40 μg total celular protein derived from quiescent (Go) cultures as well as from cells at the wound edge (E) and in the distal monolayer (M) at various times (2, 6, 12, and 24 h) post-injury, were separated by electrophoresis, blotted to nitrocellulose, and probed with rabbit antibodies to rat uPAR. Data plotted is the average of two separate densitometric assessments of cellular uPAR level changes as a function of locale (E vs. M) and time (in hours) post-wounding.

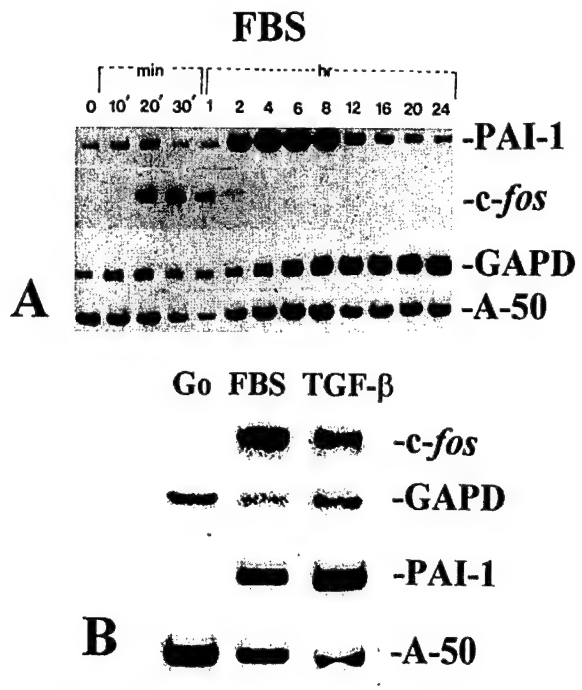

Fig. 5. Kinetics of PAI-1 and c-*fos* mRNA expression in serum-stimulated NRK cells. RNA was isolated at the times indicated after serum-stimulation of quiescent NRK cell cultures (0 = G_o cells) and Northern blots hybridized with ^{32}P-labeled probes to PAI-1, c-*fos*, GAPD, and A-50 (**A**). Under the conditions employed, addition of FBS (to a final concentration of 20%) to growth-arrested NRK cells (maintained for 3 days in serum-deficient DMEM) results in synchronous entry into S-phase at 10 to 12 h post-stimulation [Kutz et al., 1997]. PAI-1 mRNA levels are maximally increased in early- to mid-G1 and decline abruptly prior to DNA synthesis; induced c-*fos* transcripts appear restricted to the period of Go→G1 transit. GAPD transcripts increased in mid-G1 and remained elevated thereafter. A-50 expression, in contrast, was unchanged as a consequence of cell growth activation. c-*fos* and PAI-1 transcripts are also induced in quiescent (Go) NRK cells in response to TGF-β1 (1 ng/ml) in a time frame similar to that of serum-mediated expression (**B**). RNA was isolated from NRK cells for assessment of c-*fos* mRNA levels 30 min after exposure to serum or TGF-β1; PAI-1 transcripts abundance was determined after 2 h of treatment with FBS or TGF-β1. c-*fos* and PAI-1 Northern blots were normalized by re-probing with cDNAs encoding GAPD and A-50, respectively.

antibodies supershifted this complex. A molecular genetic approach was used, therefore, to down-modulate basal c-FOS levels in confluency-arrested quiescent NRK cell cultures to assess the consequences of such perturbation on induced PAI-1 synthesis and wound-dependent migration. pSV*neo-sof* transfections resulted in generation of 2 stable cell lines (NRK*sof*6, NRK-*sof*11). Although it was not possible to accurately measure constitutive c-*fos* mRNA levels in quiescent *sof*6 or *sof*11 cells due to the relatively low expression of c-*fos* in cells maintained under conditions of growth arrest, serum-inducible c-*fos* transcripts were significantly attenuated in both lines correlating with a marked reduction in PAI-1 protein synthesis (Fig. 7). This was particularly evident in *sof*6 cells. Although low levels of PAI-1 were detectable in the secreted protein complement of *sof*6 and *sof*11 cells, PAI-1 was completely absent in the SAP fraction of both lines suggesting that, like the 4HH line [Providence et al., 2000], these cells are functionally deficient. PAI-1 transcript abundance in NRK*sof*6 populations was <20% of control *neo* vector transfectants or parental cells; SDS/PAGE analysis of secreted and SAP fraction proteins indicated a specificity for pSV*neo-sof*-directed PAI-1 down-regulation (Fig. 7). Decreased PAI-1 induction, moreover, strongly corelated with inhibition of both basal and growth factor (i.e., FBS, TGF-β1)-stimulated injury repair (Table I).

Since monolayer wound closure and PAI-1 induction, even in response to a potent stimulus as serum, was attenuated in *sof*6 cells, it was important to identify potential AP-1 sites that might function in serum-stimu-

TABLE I. Inhibition of Serum- and TGF-β1-Stimulated Monolayer Wound Repair by *sof6* Transfectants*

Cell line	Culture conditions	% Injury closure
NRK parental	FBS-free	42 ± 3
	FBS	80 ± 4
	TGF-β1	69 ± 2
Neo	FBS-free	45 ± 4
	FBS	82 ± 3
	TGF-β1	71 ± 5
sof6	FBS-free	9 ± 2
	FBS	15 ± 2
	TGF-β1	12 ± 4

*Parental NRK, SV*neo-sof*, or control *neo* transfectant cell cultures were maintained in serum-free DMEM for 3 days. Prior to injury with the apex of a plastic scraper to create a 1.4-mm gap, the medium was changed to fresh DMEM without serum (to assess basal migration) or to DMEM supplemented with 10% FBS or 1 ng/ml TGF-β1 (to assess stimulated migration). Extent of wound repair was quantified 18 h later using a calibrated ocular grid. % injury closure represents the mean ± standard deviation of 20 measurements for each of 3 cultures.

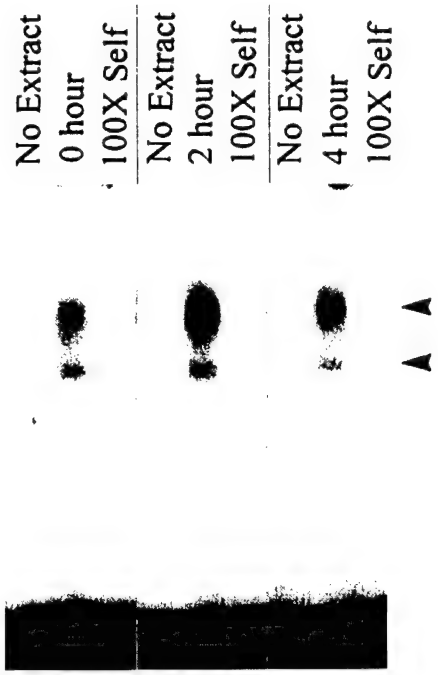

Fig. 6. Mobility shift analysis. A double-stranded deoxyoligonucleotide construct containing a consensus AP-1 sequence (5′-dTTCCGGCTGACTCATCAAGCG-3′) was end-labeled, incubated with 20 μg nuclear extract (from quiescent, 2- or 4-h serum-stimulated NRK cells) in the presence or absence of 100-fold excess unlabeled AP-1 deoxyoligonucleotide (self) and complexes (indicated by *arrowheads*) resolved on Tris-glycine gels.

lated PAI-1 gene expression. PAI-1 promoter-CAT reporter assays were designed, therefore, within the real-time of inducible expression (i.e., 4 h after addition of fresh serum) to identify responsive sequences. Progressive 5′ truncations from nt −1,230 to −266 resulted in loss of serum-activated *cis*-acting elements at each stage while repressive sequences appear to reside between nt −266 and −162. In the absence of this repressive influence, a region from −533 to −764 bp functioned as a significant (9-fold) enhancer relative to the corresponding quiescent control cells (Table II).There was no difference in the levels of CAT mRNA in the quiescent cultures among each of the constructs when compared independently. Sequence analysis of this −533 to −764 bp serum-responsive region (SRR) revealed several near-consensus motifs previously implicated as protein binding sites in other cell systems including an AP-1 site at nt −654 to −660. The AP-1-like element located within the SRR of rat PAI-1 gene (TGACACA) shares 86% homology with the consensus AP-1 (TGACTCA) heptanucleotide and is identical in sequence and approximate position to an AP-1 site in the highly homologous −800 to −595 bp region of the human PAI-1 gene. Deletion of a block of 37 nucleotides containing this AP-1 motif in the human gene is sufficient to ablate growth factor-induced expression [Keeton et al., 1991].

DISCUSSION

Proliferation confined to a relatively narrow region proximal to the injury site is required to maintain the integrity of the migrating front in wounded renal epithelial monolayers since inhibition of DNA synthesis (as with hydroxyurea) affects the cohesiveness of the motile epithelium (although migratory ability remains unaffected) [Providence et al., 2000]. Expression of PAI-1 and, to a lesser extent, uPAR, is also restricted to cells immediately bordering the wound bed and involved in the repair process. A similar functional compartmentalization occurs as part of the healing response in vivo, suggesting that several important aspects of injury resolution (i.e., regional uPAR/PAI-1 expression, spatial/temporal distinctions between the motile and proliferative phenotypes) that are evident in vivo [e.g., Pepper et al., 1993; Reidy et al., 1995; Romer et al., 1991, 1994; Zahm et al., 1997] are recapitulated during migration of NRK epithelial cells into the scrape-denuded areas of an injured monolayer. The mechanism underlying the effective proliferative inhibition in cells constituting the migrating front is not known but autocrine production of TGF-β, a stimulator of NRK locomotion and growth inhibitor (Kutz et al., unpublished data), by cells at the wound edge [Song et al., 2000] is a likely possibility. Motile cells also express high levels of uPAR and both intact pro-uPA and fragments of uPA that are capable of receptor-binding markedly reduced in vitro HL-60 cell proliferation [Howell et al., 1994].

PAI-1 expression is an early response to trauma and necessary for effective repair of monolayer wounds

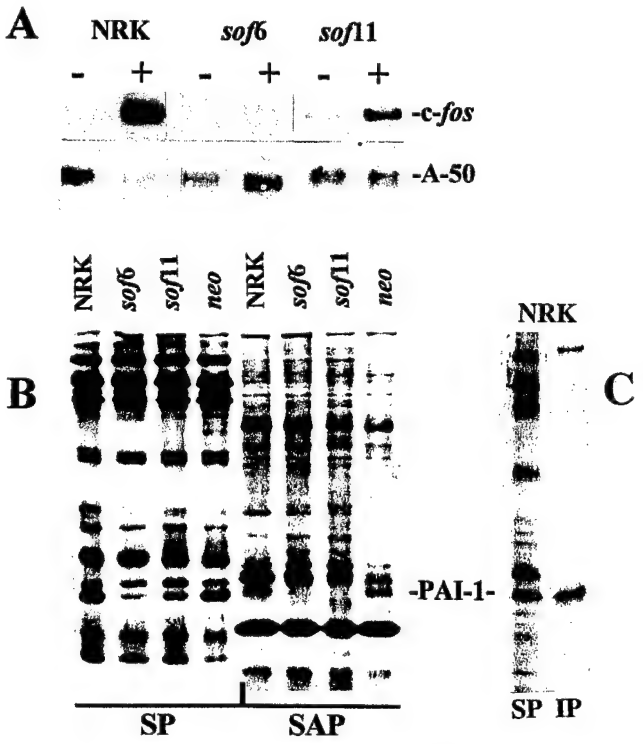

Fig. 7. Attenuation of serum-induced c-*fos* and PAI-1 expression in NRK *sof*6 and NRK *sof*11 cells. Growth-arrested NRK parental, *sof*6 and *sof*11 cells were maintained in serum-deficient medium (—) or stimulated for 30 min with serum (20%, final concentration; +). RNA was extracted and Northern blots hybridized with ^{32}P-labeled probes to c-*fos* and A-50 (**A**). Serum-induced c-*fos* mRNA abundance in *sof*6 and *sof*11 transfectants was ≤5 and 20% that of parental (NRK) controls, respectively. Electrophoretic analysis of ^{35}S-methionine-labeled secreted (SP) and SAP fraction proteins indicated that the *sof*6 and *sof*11 cell lines were also deficient in PAI-1 synthesis as evidenced by the decreased SP fraction PAI-1 levels and the complete absence of SAP fraction PAI-1 protein (**B**). PAI-1 immunoprecipitated (IP) from the SP fraction of serum-stimulated parental NRK cells (**C**) served to identify the banding position of this protein in electrophoretic separations of metabolically-labeled SP and SAP fraction proteins.

TABLE II. Relative Transcription Activity of CAT Reporter Plasmids*

Construct	CAT mRNA abundance
rPAI-CAT(−1230)	9.25 ± 4.27
rPAI-CAT(−764)	4.51 ± 1.66
rPAI-CAT(−528)	1.30 ± 0.21
rPAI-CAT(−266)	0.69 ± 0.10
rPAI-CAT(−162)	1.41 ± 1.11
p764-CAT(del 162-533)	9.23 ± 5.39

*NRK cells were transfected with the indicated plasmid DNA. Growth-arrested cultures (see Materials and Methods) were allowed to remain quiescent or serum-stimulated for 4 h. RNA was isolated and plasmid DNA extracted by the HIRT procedure. Dot-blot hybridization with a ^{32}P-labeled CAT-specific probe was used to quantify CAT mRNA and plasmid DNA abundance. CAT mRNA levels (normalized to the corresponding HIRT signal) are expressed relative to quiescent values for the same construct. Data represents the mean ± standard error for 4–8 determinations.

[Providence et al., 2000]. PAI-1 is also critical for invasive growth in vivo [Liu et al., 1995; Bajou et al., 1998]. The ability of this SERPIN to stimulate or inhibit cell motility is likely dependent, therefore, on the level and focalization of uPA activity, the composition of the ECM, the integrin complement of the cell, and the time course as well as amplitude of PAI-1 expression [Kjoller et al., 1997]. While enhanced uPA/plasmin activity is important for cellular invasion through complex matrices [Liu et al., 1995; Meissauer et al., 1991; Kawada and Umezawa, 1995], uPA activity is apparently not required for keratinocyte locomotion in vitro although uPA is required for fibrin clearance and reepithelialization of incisional injuries in vivo [Bugge et al., 1996]. Occupancy of uPAR by uPA, but not uPA catalytic activity, facilitates wound-responsive endothelial cell motility [Sato and Rifkin, 1988; Pepper et al., 1987; Ando and Jensen, 1996; Okedon et al., 1992]. uPA function in migration across a denuded zone, therefore, may be cell type-related and likely involves the differential utilization of uPA/uPAR vs. vitronectin/integrin targets as PAI-1-sensitive motors [e.g., Chapman, 1997; Loskutoff et al., 1999]. Subcellular targeting of de novo-synthesized PAI-1 to the cellular undersurface in close proximity to focal contact sites [Kutz et al., 1997], moreover, can influence uPA-dependent proteolysis and cell attachment, the latter as a consequence of interactions between the uPA/PAI-1/uPAR system and vitronectin or between PAI-1 and vitronectin/αv integrins [Loskutoff et al., 1999]. The associated changes in the temporal expression [Providence et al., 2000] and site-specific localization [Kutz et al., 1997] of PAI-1, moreover, would likely influence the stability of both pre-existing and newly formed cell-to-ECM adhesive complexes [Ciambrone and McKeown-Longo, 1990] consistently modulating, thereby, cellular migratory traits over the time course of injury repair [Blasi, 1996]. Affinity differential estimates suggest that PAI-1 may effectively dissociate bound vitronectin from the uPAR, detaching cells that utilize uPAR as a matrix anchor from a vitronectin substrate [Deng et al., 1996; Loskutoff et al., 1999]. uPAR-associated uPA/PAI-1 complexes, furthermore, are internalized by endocytosis promoting receptor recycling [Andreasen et al., 1997; Blasi, 1996] and, thereby, vitronectin-dependent cell movement. Alternatively, PAI-1 may directly inhibit αv integrin-mediated attachment to vitronectin by blocking accessibility to the RGD sequence located proximal to the uPAR binding site [Steffansson and Lawrence, 1996; Loskutoff et al., 1999].

Vector-driven c-*fos* down-regulation in NRK cells resulted in significant decreases in both PAI-1 expression

and basal as well as growth factor-stimulated cell locomotion. Similarly, c-*fos* expression has been linked to migration in periodontal ligament cells and loss, or even partial inhibition, of c-*fos* induction correlated with a marked decline in motility [Asahara et al., 1999; Xi et al., 1999]. *sof*6 cells have a reduced proliferative rate (≤60% that of parental controls), consistent with previous estimates of a 40 to 75% inhibition of cell proliferation in response to inducible synthesis of c-*fos* antisense RNA or microinjection of c-FOS antibodies [Holt et al., 1986; Riabowol et al., 1988]. This growth rate difference was unrelated to the prominent wound healing defect characteristic of *sof*6 cells. Indeed, addition of hydroxyurea to confluent NRK cell cultures prior to wounding resulted in loss of cohesiveness between individual cells (likely due to suppression of the proliferative cohort that supports the migrating front) but did not affect migration into the denuded zone [Providence et al., 2000]. FOS may contribute to PAI-1 gene control via transcriptional activation by AP-1 sites [e.g., Keeton et al., 1991; Descheemaeker et al., 1992] or by stimulation of the overall growth program. While the PAI-1 gene has a number of AP-1-like motifs [Bruzdzinski et al., 1990], one such sequence maps to nt −654 to −660 within the SRR. This SRR AP-1 element (TGACACA) is identical in sequence and in a similar position to an AP-1 site at nt −711 to −717 of the human PAI-1 gene. Deletion of nt −703 to −740 (and, therefore, this AP-1 motif) in a human PAI-1 promoter-driven reporter construct essentially eliminates growth factor-induced expression [Keeton et al., 1991]. While previous analyses suggest a general involvement of AP-1 in growth factor-initiated PAI-1 transcription in various cell types [Keeton et al., 1991; Descheemaeker et al., 1992; Johnson et al., 1992], molecular events underlying PAI-1 synthesis during the specific conditions of injury repair are unknown and the precise function of the SRR AP-1 site remain to be determined. Clearly, however, proteins that recognize the AP-1 motif (including c-FOS) are present in growth-arrested NRK cells. Since c-*fos* antisense constructs effectively down-regulate both induced PAI-1 expression and wound-associated cellular migration, and PAI-1 synthesis is important for the injury-initiated motile response in NRK cells [Providence et al., 2000], AP-1 factors are likely crucial to the overall regulation of the program of tissue repair.

ACKNOWLEDGMENTS

Supported by Public Health Service grants DK46272 and GM42461 from the National Institutes of Health and grant DAMD17-98-1-8015 from the Department of the Army. The assistance of Aftab Ahmed in generation of cell lines is gratefully acknowledged.

REFERENCES

Almendral J, Sommer D, MacDonald-Bravo H, Burckhardt J, Perera J, Bravo R. 1988. Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol Cell Biol 8:2140–2148.

Ando Y, Jensen PJ. 1996. Protein kinase C mediates up-regulation of urokinase and its receptor in the migrating keratinocytes of wounded cultures, but urokinase is not required for movement across a substratum in vitro. J Cell Physiol 167:500–511.

Andreasen PA, Kjoller L, Christensen L, Duffy MJ. 1997. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer 72:1–22.

Arts J, Grimbergen J, Bosma PJ, Rahmsdorf HJ, Kooistra T. 1996. Role of c-jun and proximal phorbol 12-myristate-13-acetate-(PMA)-responsive elements in the regulation of basal and PMA-stimulated plasminogen-activator inhibitor 1 gene expression in HepG2. Eur J Biochem 241:393–402.

Asahara Y, Nishimura F, Arai H, Kurihara H, Takashiba S, Murayama Y. 1999. Chemotactic response of periodontal ligament cells decreases with donor age: association with reduced expression of c-*fos*. Oral Dis 5:337–343.

Bajou K, Noel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D, Foidart JM. 1998. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat Med 4:923–928.

Bereiter-Hahn J. 1984. Epidermal cell migration and wound repair. In: Biology of the Integument, Vol 2: Vertebrates. Berlin: Springer-Verlag, p 443–471.

Blasi F. 1996. The urokinase receptor and cell migration. Semin Thromb Hemost 22:513–516.

Boehm JR, Kutz SM, Sage EH, Staiano-Coico L, Higgins PJ. 1999. Growth state-dependent regulation of plasminogen activator inhibitor type-1 gene expression during epithelial cell stimulation by serum and transforming growth factor-β1. J Cell Physiol 181:91–106.

Bruzdzinski CJ, Riordan-Johnson M, Nordby EC, Suter SM, Gelehrter TD. 1990. Isolation and characterization of the rat plasminogen activator inhibitor-1 gene. J Biol Chem 265:2078–2085.

Bugge TH, Flick MJ, Danton MJS, Daugherty CC, Romer J, Dano K, Carmeliet P, Collen D, Degen JL. 1996. Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci USA 93:5899–5904.

Burger C, Wick M, Bursselbach S, Muller R. 1994. Differential induction of 'metabolic genes' after mitogenic stimulation and during normal cell cycle progression. J Cell Sci 107:241–252.

Chapman HA. 1997. Plasminogen activators, integrins, the coordinated regulation of cell adhesion and migration. Curr Opin Cell Biol 9:714–724.

Ciambrone GJ, McKeown-Longo PJ. 1990. Plasminogen activator inhibitor type 1 stabilizes vitronectin-dependent adhesions in HT-1080 cells. J Cell Biol 111:2183–2195.

Deng G, Curriden SA, Wang S, Rosenberg S, Loskutoff DJ. 1996. Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? J Cell Biol 134:1563–1571.

Descheemaeker K, Wyns S, Nelles L, Auwerx J, Ny T, Collen D. 1992. Interaction of AP-1, AP-2, and Sp1-like proteins with two distinct sites in the upstream regulatory region of the plasminogen activator inhibitor-1 gene mediates the phorbol 12-myristate 13-acetate response. J Biol Chem 267:15086–15091.

Edwards SA, Rundell AY, Adamson ED. 1988. Expression of c-*fos* antisense RNA inhibits the differentiation of F9 cells to parietal endoderm. Dev Biol 129:91–102.

Felts SJ, Stang MT, Getz MJ. 1997. A c-Fos- and E1A-interacting component of the tissue factor basal promoter complex mediates synergistic activation of transcription by transforming growth factor-β1. Oncogene 14:1679–1685.

Folkman J, Moscona A. 1978. Role of cell shape in growth control. Nature 273:345–349.

Greenwood JA, Murphy-Ullrich JE. 1998. Signaling of de-adhesion in cellular regulation and motility. Microsc Res Tech 43:420–432.

Grimaldi G, DiFiore P, Locatelli, EK, Falco J, Blasi F. 1986. Modulation of urokinase plasminogen activator gene expression during the transition from quiescent to proliferative state in normal mouse cells. EMBO J 5:855–861.

Guadagno T, Ohtsubo M, Roberts J, Assoian, R. 1993. A link between cyclin A expression and adhesion-dependent cell cycle progression. Science 262:1572–1575.

Higgins PJ, Ryan MP, Zeheb R, Gelehrter TD, Chaudhari P. 1990. P52 induction by cytochalasin D in rat kidney fibroblasts: homologies between p52 and plasminogen activator inhibitor type-1. J Cell Physiol 143:321–329.

Holt JT, Gopal TV, Moulton, AD, Nienhuis, AW. 1986. Inducible production of c-*fos* antisense RNA inhibits 3T3 cell proliferation. Proc Natl Acad Sci 83:4794–4798.

Howell AL, Hunt JA, James TW, Mazar A, Henkin J, Zacharski LR. 1994. Urokinase inhibits HL-60 cell proliferation in vitro. Blood Coagul Fibrinolysis 5:445–453.

Jin G, Howe PH. 1999. Transforming growth factor β regulates clusterin gene expression via modulation of transcription factor c-Fos. Eur J Biochem 263:534–542.

Johnson M, Bruzdzinski C, Winograd S, Gelerhter TD. 1992. Regulatory sequences and protein-binding sites involved in the expression of the rat plasminogen activator inhibitor-1 gene. J Biol Chem 267:12202–12210.

Kanse SM, Kost C, Wilhelm OG, Andreasen PA, Preissner KT. 1996. The urokinase receptor is a major vitronectin-binding protein on endothelial cells. Exp Cell Res 224:344–353.

Kato GJ, Dang CV. 1992. Function of the c-Myc oncoprotein. FASEB J 6:3065–3072.

Kawada M, Umezawa K. 1995. Suppression of the in vitro invasion of human fibrosarcoma cells by a leupeptin analogue inhibits the urokinase-plasmin system. Biochem Biophys Res Comm 209: 25–30.

Keeton M, Curridan S, van Zonneveld A, Loskutoff, D. 1991. Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor β. J Biol Chem 266:23048–23052.

Kjoller L, Kanse SM, Kirkegaard T, Rodenburg KW, Ronne E, Goodman SL, Preissner KT, Ossowski L, Andreasen PA. 1997. Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. Exp Cell Res 232:420–429.

Kutz SM, Nickey S, White LA, Higgins PJ. 1997. Induced PAI-1 mRNA expression and targeted protein accumulation are early G1 events in serum-stimulated rat kidney cells. J Cell Physiol 170:8–18.

Lanahan A, Williams JB, Sanders LK, Nathans D. 1992. Growth factor-induced delayed early response genes. Mol Cell Biol 12:3919–3929.

Lau LF, Nathans D. 1987. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-*fos* or c-*myc*. Proc Natl Acad Sci 84:1182–1186.

Lauffenburger DA, Horwitz AF. 1996. Cell migration: a physically integrated molecular process. Cell 84:359–369.

Liu G, Shuman MA, Cohen RL. 1995. Co-expression of urokinase, urokinase receptor and PAI-1 is necessary for optimal invasion of cultured lung cancer cells. Int J Cancer 60:501–506.

Liu C, Yao J, deBelle I, Huang RP, Adamson E, Mercola D. 1999. The transcription factor EGR-1 suppresses transformation of human fibrosarcoma HT1080 cells by coordinated induction of transforming growth factor-β1, fibronectin, and plasminogen activator inhibitor-1. J Biol Chem 274:4400–4411.

Loskutoff DJ, Curriden SA, Hu G, Deng G. 1999. Regulation of cell adhesion by PAI-1. APMIS 107:54–61.

Meissauer A, Kramer MD, Hofmann M, Erkell LJ, Jocob E, Schirrmacher V, Brunner G. 1991. Urokinase-type and tissue-type plasminogen activators are essential for in vitro invasion of human melanoma cells. Exp Cell Res 192:453–459.

Mignatti P, Rifkin DB. 2000. Nonenzymatic interactions between proteinases and the cell surface: novel roles in normal and malignant cell physiology. Adv Cancer Res 78:103–157.

Muller R, Mumberg D, Lucibello FC. 1993. Signals and genes in the control of cell-cycle progression. Biochim Biophys Acta 1155: 151–179.

Okedon LE, Sato Y, Rifkin DB. 1992. Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial migration independent of its proteolytic activity. J Cell Physiol 150:258–263.

Pawar S, Kartha S, Toback FG. 1995. Differential gene expression in migrating renal epithelial cells after wounding. J Cell Physiol 165:556–565.

Pepper MS, Vassalli JD, Montesano R, Orci L. 1987. Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. J Cell Biol 105:2535–2541.

Pepper MS, Sappino AP, Montesano R, Orci L, Vassalli JD. 1993. Plasminogen activator inhibitor-1 is induced in migrating capillary endothelial cells. J Cell Physiol 153:129–139.

Planus E, Barlovatz-Meimon G, Rogers RA, Bonavaud S, Ingber DE, Wang N. 1997. Binding of urokinase to plasmiongen activator inhibitor type-1 mediates cell adhesion and spreading. J Cell Sci 110:1091–1098.

Pollanen J, Stephens R, Vaheri A. 1991. Directed plasminogen activation at the surface of normal and malignant cells. Adv Cancer Res 57:273–328.

Providence KM, Kutz SM, Higgins PJ. 1999. Perturbation of the actin cytoskeleton induces PAI-1 gene expression in cultured epithelial cells independent of substrate anchorage. Cell Motil Cytoskeleton 42:82–93.

Providence KM, Kutz SM, Staiano-Coico L, Higgins PJ. 2000. PAI-1 gene expression is regionally induced in wounded epithelial cell monolayers and required for injury repair. J Cell Physiol 182:269–280.

Reidy M, Irwin C, Linder V. 1995. Migration of arterial wall cells. Circ Res 78:405–414.

Riabowol KT, Vosatka RJ, Ziff EB, Lamb NJ, Feramisco JR. 1988. Microinjection of *fos*-specific antibodies blocks DNA synthesis in fibroblast cells. Mol Cell Biol 8:1670–1676.

Romer J, Lund LR, Eriksen J, Ralkiaer E, Zeheb R, Gelehrter TD, Dano K, Kristensen P. 1991. Differential expression of urokinase-type plasminogen activator and its type-1 inhibitor during healing of mouse skin wounds. J Invest Dermatol 97:803–811.

Romer J, Lund LR, Kriksen J, Pyke C, Kristensen P, Dano K. 1994. The receptor for urokinase-type plasminogen activator is ex-

pressed by keratinocytes at the leading edge during re-epithelialization of mouse skin wounds. J Invest Dermatol 102:519–522.

Ryan M, Kutz S, Higgins PJ. 1996. Complex regulation of plasminogen activator inhibitor type-1 (PAI-1) gene expression by serum and substrate adhesion. Biochem J 314:1041–1046.

Ryseck R, MacDonald-Bravo H, Zerial M, Bravo R. 1989. Coordinate induction of fibronectin, fibronectin receptor, tropomyosin, and actin genes in serum-stimulated fibroblasts. Exp Cell Res 180: 537–545.

Sato Y, Rifkin DB. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture. J Cell Biol 109:309–315.

Song QH, Singh RP, Richardson TP, Nugent MA, Trinkaus-Randall V. 2000. Transforming growth factor-beta1 expression in cultured corneal fibroblasts in response to injury. J Cell Biochem 77:186–199.

Schneidermann J, Sawdey M, Craig H, Thinnes T, Bordui G, Loskutoff DJ. 1993. Type 1 plasminogen activator inhibitor gene expression folowing partial hepatectomy. Am J Pathol 143: 753–762.

Stefansson S, Lawrence DA. 1996. The serpin PAI-1 inhibits cell migration by blocking integrin avb5 binding to vitronectin. Nature 383:441–443.

Thornton A, Bruzdzinski C, Raper S, Gelehrter TD. 1994. Plasminogen activator inhibitor-1 is an immediate early response gene in regenerating rat liver. Cancer Res 54:1337–1343.

Verrier B, Muller D, Bravo R, Muller R. 1986. Wounding a fibroblast monolayer results in the rapid induction of the c-fos proto-oncogene. EMBO J 5:913–917.

Vincent S, Marty L, LeGallic L, Jeanteur P, Fort P. 1993. Characterization of late response genes sequentially expressed during renewed growth of fibroblast cells. Oncogene 8:1603–1610.

Waltz DA, Natkin LR, Fujita RM, Wei Y, Chapman HA. 1997. Plasmin and plasminogen activator inhibitor type-1 promote cellular motility by regulating the interaction between the urokinase receptor and vitronectin. J Clin Invest 100:58–67.

Xi XP, Graf K, Goetze S, Fleck E, Hsueh WA, Law RE. 1999. Central role of the MAPK pathway in angII-mediated DNA synthesis and migration in rat vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 19:73–82.

Zahm JM, Kaplan H, Herar AL, Doriot F, Pierrot D, Somelette P, Puchelle E. 1997. Cell Migration and proliferation during the in vitro wound repair of the respiratory epithelium. Cell Motil Cytoskeleton 37:33–43.

Experimental Cell Research **260,** 127–135 (2000)
doi:10.1006/excr.2000.5001, available online at http://www.idealibrary.com on IDEAL®

Growth State-Dependent Binding of USF-1 to a Proximal Promoter E Box Element in the Rat Plasminogen Activator Inhibitor Type 1 Gene

Lisa A. White,* Carolyn Bruzdzinski,† Stacie M. Kutz,* Thomas D. Gelehrter,‡ and Paul J. Higgins*,[1]

Center for Cell Biology & Cancer Research, Albany Medical College, Albany, New York 12208; †Center for Molecular Biology of Oral Diseases, University of Illinois, Chicago, Illinois 60612; and ‡Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan 48109

Induced PAI-1 gene expression in renal epithelial (NRK-52E, clone EC-1) cells occurs as part of the immediate-early response to serum. PAI-1 transcripts are maximally expressed early in G_1 (within 4 h of serum addition to quiescent EC-1 cells) and then subsequently decline to basal levels prior to entry into DNA synthetic phase. Comparative analysis of PAI-1 mRNA abundance and *de novo*-synthesized thiolated RNA in quiescent cells, as well as at 4 h (early G_1) and 20 h (late G_2) postserum addition, in conjunction with RNA decay measurements indicated that PAI-1 gene regulation upon growth activation was predominantly transcriptional. An E box motif (CACGTG), important in the induced expression of some growth state-dependent genes, mapped to nucleotides −160 to −165 upstream of the transcription start site in the PAI-1 proximal promoter. Mobility-shift assessments, using a 18-bp deoxyoligonucleotide construct containing the E box within the context of PAI-1-specific flanking sequences, confirmed binding of EC-1 nuclear protein(s) to this probe and, specifically, to the E box hexanucleotide site. The specificity of this protein-probe interaction was verified by competition analyses with double-stranded DNA constructs that included E box deoxyoligonucleotides with non-PAI-1 flanking bases, mutant E box sequences incapable of binding NRK nuclear proteins, and unrelated (i.e., AP-1) target motifs. Extract immunodepletion and supershift/complex-blocking experiments identified one PAI-1 E box-binding protein to be upstream stimulatory factor-1 (USF-1), a member of the HLH family of transcription factors. Mutation of the CACGTG site to TCCGTG in an 18-bp PAI-1 probe inhibited the formation of USF-1-containing complexes confirming that an intact E box motif at −160 to −165 bp in the PAI-1 promoter and, in particular, the CA residues at −165 and −164 are essential for USF-1 binding. Incorporation of this 2 bp change into a reporter construct containing 764 bp of the proximal PAI-1 "promoter" ligated to a CAT gene effectively reduced (by 74%) CAT activity in cycling cells. An intact E box motif at nucleotides −160 to −165 in the PAI-1 promoter, thus, is an important functional element in the regulation of PAI-1 transcriptional activity in renal cells. © 2000 Academic Press

INTRODUCTION

Genes encoding products required for G_1 transit and entry into DNA synthetic phase are activated within a defined time frame and for a specified duration following mitogen-induced recruitment of quiescent (G_0) cells into the proliferative cycle [1–5]. Such growth-associated genes segregate among the immediate-early, delayed-early, and late response sets depending on their kinetics and mode of induction [6–8]. Immediate-early response (IER) genes are expressed either within brief windows during the G_0–G_1 transition (e.g., c-*fos*) or throughout the G_1 phase as well as by continuously cycling cells (e.g., c-*myc*) [9, 10]. A metabolic hallmark of IER genes is rapid stimulus-mediated transcription involving, at least initially, a primary (i.e., protein synthesis-independent) inductive pathway [1, 3, 5, 8]. In contrast, delayed-early and late genes usually exhibit secondary response characteristics (i.e., require protein synthesis for induction) and preferential expression during mid-to late-G_1 and S phases (e.g., T1, thymidine kinase) [11]. IER genes encode trancription factors (which target the delayed-early and late genes necessary for continued cell growth), structural proteins (that comprise the cytoskeleton, extracellular matrix, and cell-to-matrix adhesion complexes), and matrix-active proteases and their inhibitors [4, 12]. Collectively, IER gene products initiate specific changes in gene reprogramming and cytoarchitecture required for progression of activated cells through the proliferative cycle [12–15].

Prominent among the repertoire of genes activated in response to serum is that encoding the type 1 inhibitor of plasminogen activator (PAI-1) [15–18]. Synthe-

[1] To whom correspondence and reprint requests should be addressed at the Center for Cell Biology & Cancer Research, Albany Medical College (MC165), 47 New Scotland Avenue, Albany, NY 12208. Fax: (518) 262-5696. E-mail: higginp@mail.amc.edu.

0014-4827/00 $35.00

sis of PAI-1 mRNA occurs rapidly and transiently upon addition of serum to quiescent cells [15, 17, 19] as well as during compensatory regeneration following tissue injury [20, 21]. PAI-1 complexes with urokinase and tissue-type plasminogen activators limiting, thereby, the generation of plasmin, a broad spectrum protease involved in fibrin surveillance and extracellular matrix turnover [22, 23]. PAI-1 may function, therefore, as part of a global program of tissue remodeling/wound repair or, more specifically, within the context of the growth-activated phenotype, to create an adhesive environment permissive for anchorage-dependent cell proliferation and/or migration [16, 17, 24, 25]. Serum-induced PAI-1 transcription has IER kinetics and occurs concomitantly with synchronous G_0 exit and entry of stimulated cells into a cycling G_1 condition [17, 18]. PAI-1 mRNA transcript abundance is maximal 4 h postserum exposure; late in G_1 phase, however, secondary events predominate and likely mediate a rapid decline in mRNA abundance [15]. Since the available data suggest that cell cycle phase-specific constraints are superimposed on growth state-dependent transcriptional regulation of the PAI-1 gene during the $G_0 \rightarrow G_1$ transition [15], we used a synchronous model of cell growth activation to study molecular events associated with induced PAI-1 gene expression.

MATERIALS AND METHODS

Cell culture. Early passage renal epithelial (NRK-52E, clone EC-1) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). To initiate a state of quiescence, 70–80% confluent cultures were washed with Ca^{2+}/Mg^{2+}-free HBSS and the media was changed to serum-free DMEM. Cells were synchronously recruited into the growth cycle after 3 days of serum deprivation by the addition of FBS to a final concentration of 20%.

RNA isolation and Northern blot analysis. RNA was isolated under conditions described in the text, separated by electrophoresis, transferred onto Nytran membranes by capillary action using 10X SSPE (1.8 M NaCl, 100 mM $NaPO_4$, pH 7.4, 10 mM ethylenediaminetetraacetic acid [EDTA]), and UV-crosslinked. Blots were incubated at 42°C for 2 h in 50% formamide, 5X Denhardt's reagent, 1% sodium dodecyl sulfate (SDS), 100 μg/ml salmon sperm DNA, 5X SSPE buffer and then hybridized at 42°C for 16 h with 5×10^6 cpm [^{32}P]dCTP-labeled cDNA probes to rat PAI-1, glyceraldehyde phosphate dehydrogenase (GAPD), actin [15], or A-50 (an RNA transcript the abundance of which does not change as a function of serum stimulation [15]) in 50% formamide, 2.5X Denhardt's reagent, 1% SDS, 100 μg/ml salmon sperm DNA, 5X SSPE, and 10% dextran sulfate. Membranes were washed three times (15 min each) at 42°C in 0.1% SSPE/0.1% SDS followed by three washes at 55°C and exposed to X-OMAT AR-5 film.

Analysis of de novo-synthesized transcripts. Thiouridine (4-TU; 100 μM) [26–28] was added to serum-stimulated cultures 2 h prior to RNA isolation. Cellular RNA (200 μg) was incubated at 55°C for 10 min in 0.5 ml buffer A (50 mM sodium acetate, 0.1% SDS, 0.15 M NaCl, 4 mM EDTA) and then batch-absorbed in the dark to 3 ml of Affigel-501 (Bio-Rad, Melville, NY) in 1.2 ml of buffer A for 2 h on ice. Affigel-501/RNA slurry columns were washed with 2.4 ml buffer A followed by 2.4 ml of buffer A containing 0.5 M NaCl to remove nonspecifically bound RNA and thiol-containing transcripts eluted with 2.4 ml buffer A containing 10 mM 2-mercaptoethanol. RNA was precipitated with 0.1 M sodium acetate and 65% absolute ethanol (final concentrations) at −20°C for 16 h, washed with 0.5 ml 75% ethanol, and resuspended in 0.1 mM EDTA. For PAI-1 mRNA half-life measurements, actinomycin D (5 μg/ml) or 5,6 dichlorobenzimidazole riboside (50 μg/ml) was added independently to cell cultures 4 h postserum stimulation. RNA was isolated at Time 0 (prior to drug addition) and at hourly increments up to 4 h postaddition of drug (i.e., 8 h after serum stimulation).

Nuclear extracts. Quiescent and serum-stimulated cells were trypsinized, harvested by centrifugation, resuspended in 400 μl of cold buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT), placed on ice for 15 min, and then vortexed for 10 s after the addition of 25 μl 10% Nonidet NP-40. Lysates were centrifuged for 30 s at 14,000*g;* nuclei were resuspended in 50 μl of cold lysis buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT containing leupeptin, aprotinin, chymostatin, pepstatin A, antipain [each at a final concentration of 10 μg/ml]) and rocked at 4°C for 15 min, and extracts were clarified at 10,000*g* for 5 min.

PCR. A ^{32}P-labeled 100-bp deoxyoligonucleotide probe encompassing the PAI-1 E box (at −165 to −160 bp upstream of the transcription start sit) was PCR-generated. Primers corresponded to nucleotides −234 to −216 (5′-CAGAGATGTTCCAGTCAAG-3′) and −152 to −135 (5′-CTCCCTCCCAGTAACTTG-3′), relative to the transcription start site. The PCR consisted of 50 μl of buffer B (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 0.9 mM dGTP, dTTP, and dATP, 100 ng template plasmid (cloned insert corresponding to nucleotides −1237 to +34 of PAI-1 genomic sequence) [29], 50 pM of each primer, 50 μCi [^{32}P]dCTP (3000 Ci/mmol), and *Taq* polymerase (0.75 μl, 5 U). The PCR product was chloroform-extracted and retrieved by passage through a 30,000 MW spin column followed by electrophoresis on a 4% Tris/glycine polyacrylamide gel for 1 h at 130 V. The probe was excised from the gel and eluted in 500 μl STE (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, pH 8.0). Unlabeled PCR competitors were also generated.

Mobility-shift assay. Double-stranded deoxyoligonucleotides (3–5 pM) were incubated at 37°C for 10 min with T4 polynucleotide kinase (5–10 units/μl) in 70 mM Tris-HCl buffer, pH 7.6, containing 10 mM $MgCl_2$, 5 mM DTT, and [^{32}P]dATP (3000 Ci/mmol). Probe was purified by filtration through a 10,000 MW cellulose spin column. Constructs used were as follows (only the coding strand is indicated): PAI-1 E box, 5′-TACACA**CACGTG**TCCCAG-3′; PAI-1 mutant E box #1, 5′-TACACA**CACGGA**TCCCAG-3′; PAI-1 mutant E box #2, 5′-TACACA**TCCGTG**TCCCAG-3′; standard consensus E box, 5′-GGAAGCAGAC**CACGTG**GTCTGTGCTTCC-3′; AP-1 consensus sequence, 5′-CGCTTGA**TGACTCA**GCCGGAA-3′.

Nuclear extracts were incubated with 50,000–250,000 cpm of ^{32}P end-labeled deoxyoligonucleotides in 5X gel-shift buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.4 mg/ml dIdC). Following room temperature incubation for 20 min, gel loading buffer (25 mM Tris-HCl, pH 7.5, 0.02% bromphenol blue, 0.02% xylene cyanol, 4% glycerol) was added. Complexes were separated on TBE gels (5% TBE buffer [890 mM Tris-HCl, pH 8.0, 890 mM boric acid, 20 mM EDTA] containing 2.5% bis-acrylamide, 10% acrylamide, 3.1% glycerol, 0.5% TEMED, 0.75% ammonium persulfate), Tris/glycine gels (Tris/glycine buffer [50 mM Tris-HCl, 2 mM EDTA, 100 mM glycine] containing 4% polyacrylamide, 0.5% bis-acrylamide, 2.5% glycerol, 0.075% ammonium persulfate, 0.085% TEMED), or TBE 3-12% gradient gels.

UV crosslinking, immunodepletion, and supershift analyses. The PAI-1 18-bp wild-type (WT) E box deoxyoligonucleotide was ^{32}P-body-labeled by PCR using a primer set corresponding to nucleotides −170 to −164 (5′-TACACA-3′) and −152 to −158 (5′-CTGGGA-3′) and purified via a 10-kDa spin column. Nuclear extract binding

reactions were carried out in a 96-well microtiter plate for 20 min at room temperature prior to UV irradiation (4.8 to 24.0 $\mu J/cm^2$) followed by DNase-1-treatment (2 μg/ml). Sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol, 0.01% bromphenol blue) was added, and the complexes were boiled and resolved on SDS/9% polyacrylamide slab gels (9% acrylamide, 0.24 % bis-acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.03% ammonium persulfate, 0.025% *N,N,N′,N′*-tetramethylethylenediamine). For immunodepletions, extracts (40 μg) were brought to a final volume of 30 μl with Ca^{2+}/Mg^{2+}-free PBS; antibodies (Chemicon or Santa Cruz Biotechnology) were used at 1 μg/reaction (MYC [AP-132]; MAX [c-17 and c-124]; MYC [c-33]; MAD [c-19]; USF-1 [c-20]; USF-2 [N-18]; c-FOS [4-1D-G]. In control reactions, the specific Ig was replaced with nonimmune Ig, with antibody of the appropriate isotype to an irrelevant antigen (i.e., annexin), or bovine serum albumin (BSA) at a final concentration of 1 μg/ml. Mixtures were placed at 4°C for 1 h and 5 μl protein G beads added for an additional incubation at 4°C for 1 h with moderate shaking. After centrifugation at 10,000*g* at 4°C, 10-μl supernatant aliquots were used in mobility shifts. Antibodies (1 to 2 μg per reaction) were either added to the formed extract protein/^{32}P-labeled DNA probe complexes and maintained at room temperature for 20 min prior to electrophoresis or incubated with nuclear extracts prior to probe addition for supershift and blocking assays, respectively.

Site-directed mutagensis of the PAI-1 E box. The E box motif (CACGTG) was mutated to TCCGTG in the PAI-1 genomic sequence plasmid as well as in the rPAI-CAT(−764) vector, which contains 764 bp of proximal PAI-1 5′ flanking genomic sequence cloned upstream of a CAT reporter gene, using the QuickChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA). The PCR contained 5 μl 10X reaction buffer [100 mM KCl, 60 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 1% Triton X-100, 100 μg/ml nuclease-free bovine serum albumin], 5–50 ng of double-strandard DNA template, 1.25 ng appropriate mutagenizing primers, 1 μl of a 10 mM stock of dNTP mix (2.5 mM each NTP), and 1 μl of PFU DNA polymerase (2.5 U/μl). The reaction was overlaid with 30 μl mineral oil and amplified. Following PCR, 1 μl Dpn I (10 U/μl) was added and the reaction mixture incubated at 37°C for 1–2 h to digest the parental DNA plasmid. Recovered mutant plasmids were transformed into XL-1 Blue supercompetent bacteria. Derived mutations were confirmed by loss of E box-specific endonuclease sensitivity, using 1 μl BbrPI (10 U/μl) which cleaves the palindromic E box motif CAC ↓ GTG, and by sequencing.

Transfection. Cells were washed twice in antibiotic- and serum-free DMEM; LipofectAMINE (28 μl/dish)-CAT reporter DNA (4 μg/dish) complexes were added (optimal concentrations of each determined by titration); and cultures were incubated at 37°C for 5 h prior to the addition of DMEM/10% FBS. The following day, the cells were trypsinized and subcultured in serum-containing DMEM; 24 h later, cells were harvested; aliquots were placed in two microfuge tubes; the cells were collected at 10,000*g* and washed with CMF-HBSS. One tube was used to prepare whole-cell extracts (for CAT activity assessments by thin-layer chromatography) while the other was used for HIRT extraction (for calculation of transfection efficiency).

CAT assays. Cells were resuspended in 50 μl of 0.25 M Tris-HCl, pH 7.8, vortexed, and lysed by three cycles of freeze-thawing, and debris was removed by centrifugation at 10,000*g* for 5 min at 4°C. The supernatant was collected, heated at 65°C for 10 min, and incubated at 37°C in a mixture containing 10 μl [^{14}C]chloramphenicol (specific activity 59.5 mCi/mmol), 50 μl 1 M Tris-HCl, and 20 μl 3.5 mg/ml acetyl-CoA; after 2 h, 1 ml ethyl acetate was added, tubes were vortexed and centrifuged at room temperature for 5 min, and the upper (aqueous) phase (900 μl) was removed and placed in a separate tube. The samples were dried using a DNA 110 Speed Vac, resuspended in ethyl acetate (15 μl), spotted onto KF6 silica-coated plates (Whatman Laboratories, Clifton, NJ), and chromatographed in 190 ml chloroform/10 ml ethanol for 2 h.

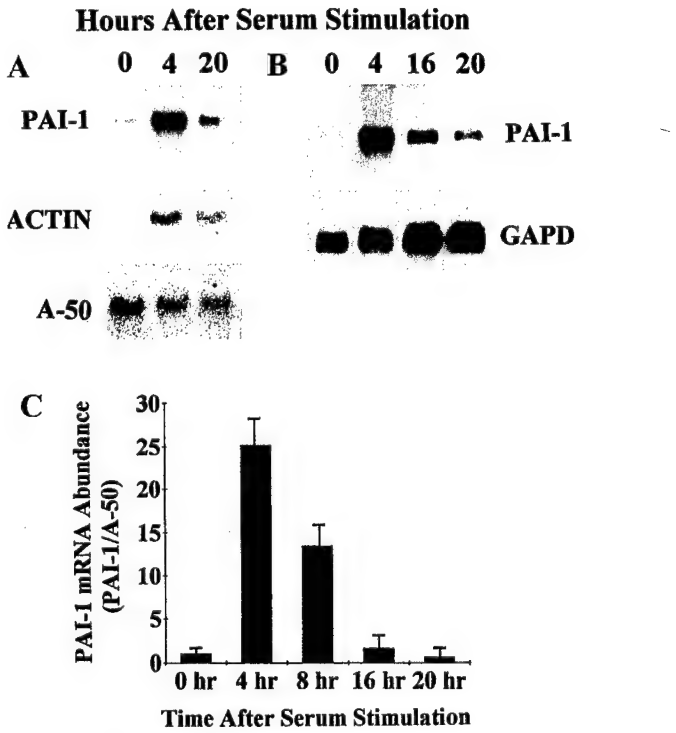

FIG. 1. Kinetics of induced PAI-1, actin, A-50, and GAPD expression in EC-1 cells as a function of time after serum stimulation. PAI-1 mRNA levels were maximal 4 h postserum addition to quiescent cells and declined by 16–20 h (A). GAPD transcripts, in contrast, progressively increased during synchronous transit of stimulated EC-1 cells through the division cycle whereas A-50 mRNA levels remained unchanged (A, B). Data in C represent the mean ± standard deviation of PAI-1 mRNA abundance normalized to A-50 signal derived from scan analysis of blots from three separate experiments.

HIRT extraction. Cells were resuspended in 300 μl 0.6% SDS and 10 mM EDTA and maintained at room temperature for 15 min, and NaCl was added to a final concentration of 1 M; protein and high MW DNA were allowed to precipitate overnight at 4°C. After centrifugation for 30 min at 4°C, supernatants were collected and incubated at 37°C following addition of Rnase (5 μl of a 1 μg/ml stock). Proteinase K was added (1 μg of a 20 μg/ml stock) for a 1-h incubation at 37°C; plasmid DNA recovered by phenol:chloroform extraction, denatured by addition of NaOH to 0.3 N, and incubated at 65°C for 1 h; 6X SSPE (400 μl) was added; DNA was transferred to Nytran membranes and UV cross-linked. Folowing incubation for 2 h at 65°C in 5X Denhardt's solution, 6X SSPE, 0.5% SDS, and 150 μg/ml heat-denatured salmon sperm DNA, a random-primed ^{32}P-labeled CAT probe was added for hybridization overnight. Blots were washed three times for 20 min each with 0.1X SSPE/0.1% SDS prior to autoradiography.

RESULTS

Addition of serum (to a final concentration of 20%) to cells previously rendered quiescent by growth factor-deprivation results in synchronous G_0 exit and entry into a cycling G_1 state [15] with concomitant induction of PAI-1 expression (Fig. 1). PAI-1 transcript abun-

dance is maximal 4 h postserum stimulation (25-fold increase in PAI-1 mRNA content relative to quiescent controls). Late in G_1 and prior to S phase (DNA synthesis occurs 10–12 h after serum addition to quiescent NRK-52E clone EC-1 cell cultures; [18]), PAI-1 mRNA levels decrease and continue to decline throughout the remainder of the growth cycle (Fig. 1C). Actin transcripts accumulated with kinetics similar to that of PAI-1. The amplitude of PAI-1 induction (i.e., relative expression in stimulated as compared to quiescent cells), however, was considerably greater (by 5-fold) than that of actin, at least at 4 h postserum addition. GAPD mRNA, in contrast, continued to increase following growth "activation" reaching a maximum (approximately 3-fold) at 20 h (Fig. 1B).

Induced PAI-1 expression during the G_0-G_1 transition occurs as part of the immediate-early response to mitogens [15, 30]. The actual mode(s) of PAI-1 gene control operative throughout the later phases of the growth cycle, however, is unknown and complicated by a duality of regulatory events involving both primary (i.e., protein synthesis-independent) and secondary (i.e., protein synthesis-dependent) mechanisms which merge in approximately mid-G_1 [18]. Previous kinetic assessments indicated that PAI-1 transcriptional activity increased as early as 10 min following serum addition [15]. Induction occurred in the presence of protein synthesis inhibitors although PAI-1 gene activity at the late stages of activation (i.e., 20 h postserum addition) was not addressed. Northern analysis of thiolated RNA disclosed a profile of PAI-1 and GAPD transcription that reflected steady-state mRNA levels (Fig. 2A). Due to the progressive increase GAPD transcription evident throughout the growth cycle of activated EC-1 cells, and the 3-fold differential between quiescent (Time 0) and 20 h serum-stimulated GAPD mRNA levels, A-50 hybridization signal was used to normalize PAI-1 transcriptional rate. A 16-fold increase in PAI-1 transcription (relative to quiescent cells) was evident at 4 h postserum stimulation while *de novo* expression of GAPD transcripts remained unchanged (Fig. 2B). GAPD transcription was elevated 2.5-fold over quiescent controls 20 h after serum addition closely reflecting the 3-fold increase in steady-state GAPD mRNA abundance at this same time point while *de novo* PAI-1 mRNA synthesis declined from a maximum at 4 h to relatively low levels by 20 h (Figs. 1B, 2A, and 2B). There was no significant difference either in the amount of RNA recoverable from equivalent numbers of EC-1 cells or in the percentage thiolated RNA (at the 0, 4, or 20 h time points). Such abrupt growth state-related changes in PAI-1 transcription and steady-state RNA levels necessitated an evaluation of PAI-1 transcript decay rate specifically during the period of transit from maximal (4 h) to basal (20 h) expression. Actinomycin D was added (final concentration of 5

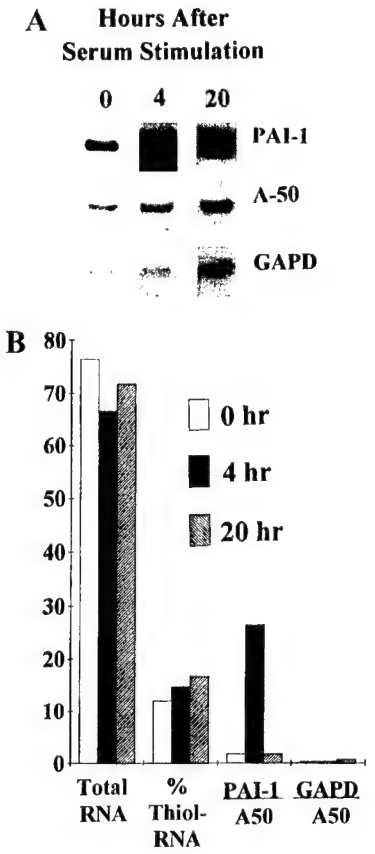

FIG. 2. Time course of serum-induced PAI-1 transcription. Northern blots of *de novo*-synthesized 4-TU-labeled RNA (A) were scanned and PAI-1 and GAPD transcript signals normalized to that of A-50 (i.e., PAI-1/A-50 and GAPD/A-50; *y* axis = arbitrary densitometric units) (B). Relative to quiescent EC-1 cultures (0 h), PAI-1 transcripts increased 16-fold at 4 h postserum addition while at 20 h PAI-1 transcription was similar to that of unstimulated cells when normalized to A-50 signal. Thiolated GAPD transcripts were maximal (2.5-fold) at 20 h postserum addition closely approximating the 20-h 3-fold increase in steady-state GAPD mRNA abundance (*y* axis for thiol-RNA = % of total RNA). Total RNA recovered at each time point (*y* axis for total RNA = μg RNA extracted from equal numbers of cells) was equivalent as were the levels of thiol-containing transcripts (B). Data shown are representative of three separate experiments.

μg/ml) to cell cultures 4 h after serum-stimulation; RNA was isolated prior to drug addition and at hourly intervals thereafter for Northern analysis. PAI-1 mRNA half-life approximated 90 min (Fig. 3), consistent with the decay rate calculated for PAI-1 transcripts in serum-stimulated NIH 3T3 fibroblasts [1]. Identical results were obtained using 5,6 dichlorobenzinidazole riboside (not shown). The kinetic profiles of steady-state PAI-1 mRNA levels, particularly at the 4 and 20 h time points, coupled with the rather short mRNA half-life and declining PAI-1 transcriptional rate are compatible with attainment of the low transcript abundance evident in the late states (i.e., 20 h postserum addition) of the cell growth cycle (Fig. 1).

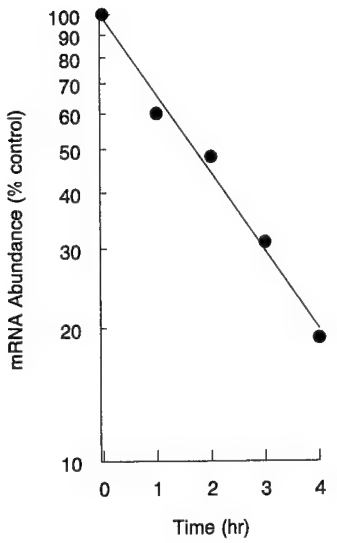

FIG. 3. Assessment of PAI-1 mRNA stability in serum-activated EC-1 cells. EC-1 cells were stimulated with serum for 4 h, actinomycin D was added, and RNA was isolated at the indicated times postinhibitor exposure. Quantitative densitometry of Northern blot signal was used to calculate a PAI-1 mRNA half-life of approximately 90 min during the early stage of transit from maximal (at 4 h postserum stimulation) to basal expression.

A search of the 5′ flanking region of the PAI-1 gene, to identify potential *cis*-acting elements involved in growth state-dependent gene expression, identified a consensus E box motif (CACGTG) at nucleotides −165 to −160 upstream of the transcriptional start site. This region is protected from DNase I digestion in growing HTC cells [29]; neither the identity of the bound proteins nor the potential growth cycle dependency of protein binding, however, was determined. In order to establish PAI-1 E box element occupancy in EC-1 cells, a ^{32}P body-labeled 100-bp segment of the PAI-1 gene (including the E box motif) was used as a probe. Preliminary findings confirmed that nuclear protein(s) present in extracts from 4 to 20 h serum-stimulated cells retarded the mobility of this PAI-1 100-bp promoter fragment (Fig. 4A). An unlabeled 18-bp deoxyoligonucleotide containing a consensus E box motif in the context of PAI-1 5′ flanking sequences effectively competed the most prominent band shift obtained with this 100-bp probe, indicating that protein(s) bound within this DNA-protein complex were specific to the E box motif and flanking nucleotides (Fig. 4A). As further evidence of specificity of this interaction, the unlabeled self 100-bp PCR DNA product competed all probe complexes formed by proteins in the 4 and 20 h extracts while an AP-1 deoxyoligonucleotide (5′-CGCTTGA**TGACTCA**GCCGGAA-3′) was ineffective (not shown). Since the 18-bp PAI-1 construct with the E box motif was a major protein-binding sequence, at least when used as a competitor, it was important to characterize this binding in more detail. Complexes were resolved in mobility-shift experiments upon incubation of nuclear extracts from 4 and 20 h FBS-stimulated cells with the ^{32}P-labeled PAI-1 E box 18-bp deoxyoligonucleotide probe; such binding activity was not as prominent in shifts developed with extracts from quiescent cells (i.e., 0 h) (Fig. 4B). This probe shift pattern was maintained when unlabeled AP-1 or mutant E box (PAI-1 mutant E box construct 1; CACGGA) deoxyoligonucleotide competitors (both in 100-fold molar ex-

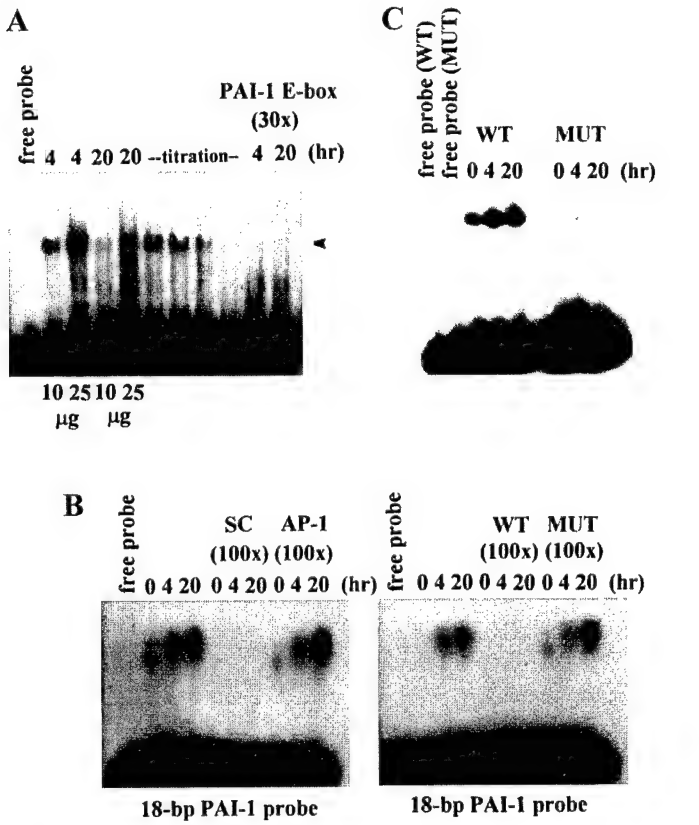

FIG. 4. Binding of nuclear factors to the PAI-1 proximal promoter E box. Resolution of the most prominently shifted complex (arrowhead in A) generated upon incubation of a^{32}P body-labeled 100-bp PAI-1 promoter fragment with nuclear protein from serum-stimulated (4 and 20 h) EC-1 cells was dependent on input nuclear extract and could be titrated by decreasing the protein content in the binding reaction. Unlabeled competing DNAs (at the indicated molar excess) included the 100-bp (self) fragment (not shown) and the 18-bp PAI-1 E box sequence (A). This same 18-bp PAI-1 E box construct was ^{32}P end-labeled and used as a probe; a standard consensus (SC) E box (CACGTG) construct with non-PAI-1-related flanking sequences competed for binding to the PAI-1 probe while an unrelated sequence (AP-1) was ineffective (B). The PAI-1 E box self-competitor (WT) eliminated probe binding while a mutant (MUT) E box (CACGGA) construct failed to inhibit complex formation between the nuclear factors and the 18-bp PAI-1-specific E box WT probe (B). As a control, the mutant E box deoxyoligonucleotide was end-labeled for use as a probe in mobility-shift reactions. The wild-type 18-bp PAI-1 E box probe (WT) produced the expected complexes when incubated with nuclear extracts from quiescent and serum-stimulated cells; the mutant (MUT) construct, in contrast, failed to produce band shifts (C) consistent with its ineffectiveness as a competing sequence (B).

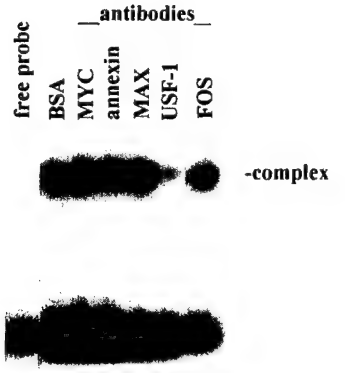

FIG. 5. Immunodepletion of 20 h FBS-stimulated nuclear extracts with antibodies to USF-1 reduces binding activity to the PAI-1 E box. Incubation of extracts with antibodies to MYC, MAX, FOS, or annexin or inclusion of BSA in the reaction mixture prior to addition of the ^{32}P-labeled 18-bp PAI-1-specific probe failed to block formation of DNA/protein complexes. Antibodies to USF-1, in contrast, effectively inhibited or substantially reduced the typical PAI-1 E box probe shift.

cess) were included in the reaction mixture. Band shifts were successfully competed, however, upon simultaneous addition of a 100-fold molar excess of an unlabeled wild-type (WT) self-competitor or a standard consensus (SC) E box construct (i.e., an E box hexanucleotide motif with non-PAI-1 flanking sequences; as under Materials and Methods) (Fig. 4B). The mutant E box competitor (CACGGA), flanked by PAI-1-specific sequences, was an unsuccessful competitor (Fig. 4B) and incapable of forming shift complexes when ^{32}P end-labeled and used as a probe in a gel-retardation assay (Fig. 4C), thus indicating a requirement (and specificity) for an intact consensus hexanucleotide E box for protein binding.

Preliminary UV-crosslinking studies, initiated to identify factors capable of complexing to the PAI-1 E box, resolved proteins of approximately 62-, 45-, and 30-kDa as bound species (not shown). Nuclear extracts from 20 h serum-stimulated EC-1 cells (which possessed prominent E box-binding activity; e.g., Fig. 4B) were subsequently incubated, therefore, with antisera to transcriptional elements with known E box-recognition specificity (i.e., the bHLH or bHLHZ proteins) and that corresponded to this mass range. Mobility-shift assays using such immunodepleted extracts resulted in the identification of USF-1, a 43-kDa CACGTG-binding protein (see Discussion), as a PAI-1 E box binding factor (Fig. 5). Supershift and combined supershift/blocking analyses confirmed at least one PAI-1 E box resident protein to be USF-1 under conditions of induced growth (Fig. 6A) as well as in constitutively growing cells (Fig. 6B). Fine analysis of the gel shift pattern, by titering levels of input nuclear protein, resolved two closely-spaced bands (particularly prominent in shifts developed with the 20 h nuclear extract). The mobility of only the upper band in this complex (single arrowhead in Fig. 6A), however, was supershifted (or formation of this complex blocked) when antibodies to USF-1 were included in the nuclear extract PAI-1 E box probe reaction, indicating the presence of USF-1 in the formed DNA-protein complexes. Addition of an antibody to USF-2, in contrast, had no effect on the pattern of probe retardation (Fig. 6B). Band-shift analysis with a second mutant E box construct confirmed the requirement for an intact WT sequence at −165 to −160 bp. The PAI-1 template plasmid (Materials and Methods) was used to create the mutation CACGTG → TCCGTG at the PAI-1 E box site (Fig. 7A). This particular mutation was designed since HLH proteins with known E box recognition activity have a conserved glutamate residue which forms hydrogen bonds with the first cytosine and adenine of the E box motif [31]. Preliminary studies indicated that this base change inhibited the formation of the characteristic E box-dependent mobility shift (e.g., Fig. 4A, arrowhead) when a 100-bp PCR-generated PAI-1 proximal promoter fragment composing the mutant E box motif was used as a probe (not shown). As binding was apparently dependent on the presence of a consensus E box motif (Fig. 4B and 4C), an 18-bp deoxyoligonucle-

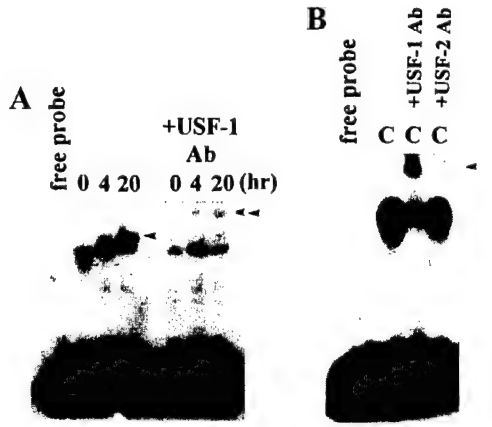

FIG. 6. Identification of USF-1 as a component of the PAI-1 E box-binding complex. Incubation of a polyclonal USF-1 antibody in reaction mixtures containing a ^{32}P-labeled 18-bp PAI-1-specific DNA probe (as in Figs. 4B and 4C) and nuclear extracts from 4 and 20 h serum-stimulated EC-1 cells produced a prominant supershift (double arrowheads in A), or effectively blocked formation, of the upper component in the shifted complex (single arrowhead in A) particularly evident at the 20-h time point (A). Since antibody was included at the time of DNA-protein addition (in A), considerable inhibition of complex formation specific for the upper band in the shift (single arrowhead in A) is also evident. The USF-1-specific supershifted band in (B) (indicated with a single arrowhead) was resolved by the addition of antibodies after formation of DNA-protein complexes. Inclusion of a polyclonal antibody to USF-2, in contrast, had no effect on migration of the probe complex. Nuclear extracts were derived from quiescent EC-1 cells (0 h) and cells stimulated with serum for 4 and 20 h, as well as from constitutively growing (c) cultures.

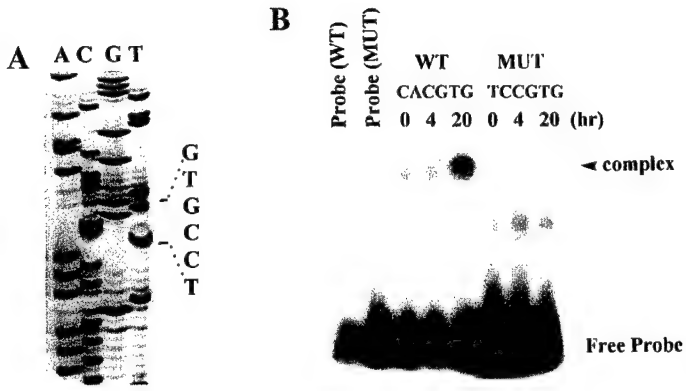

FIG. 7. Mobility-shift analysis of PAI-1 deoxyoligonucleotide probes containing wild-type and mutant E box sequences. The PAI-1 consensus E box motif (CACGTG) in the template plasmid was mutated to TCCGTG by site-directed mutagenesis (A); this base change inhibited formation of the prominent consensus E box-dependent band shift (as in Fig. 4A). Gel-retardation assays, using consensus (CACGTG) and mutant (TCCGTG) E box deoxyoligonucleotide probes (Materials and Methods), confirmed that this E box mutation abrogated binding of E box-specific nuclear factors from EC-1 cells (B).

otide bearing the same PAI-1 E box sequence substitutions (PAI-1 mutant E box construct 2; TCCGTG) as in the PCR-generated fragment was used in confirmatory mobility-shift assays. While the wild-type PAI-1 E box probe produced the expected band-retardation pattern, the mutant PAI-1 E box construct failed to bind nuclear factors indicating that the CACGTG → TCCGTG base change was not permissive for complex formation (Fig. 7B).

The mutation CACGTG → TCCGTG was also incorporated into rPAI-CAT(−764), a reporter construct containing 764 bp of PAI-1 genomic 5′ flanking sequence (including the E box at nucleotides −160 to −165) cloned upstream of a CAT gene. To assess the functional consequences of this sequence change, EC-1 cells were transfected with either the wild-type or mutant reporter plasmids. Transfectants were grown in DMEM containing 10% FBS for 24 h prior to assessment of CAT activity. This mutation reduced CAT activity in transfected cells by 74% compared to CAT levels driven by the wild-type PAI-1 promoter (Fig. 8). The difference in CAT expression between wild-type and mutant rPAI-CAT(−764) transfectants was highly significant ($P < 0.001$), indicating that an intact E box is necessary for serum-regulated PAI-1 promoter function in the EC-1 cell line.

DISCUSSION

The rapid kinetics of PAI-1 undersurface deposition following mitogenic stimulation of EC-1 cells [17, 18] and short matrix-associated half-life [32] suggest that this protein may influence cellular adhesive characteristics for a specified duration within the context of "activated" cell growth [12]. Assessment of G_1 transit (using [^{3}H]thymidine autoradiography to delineate onset of the S phase), moreover, indicated that maximal PAI-1 transcript levels are restricted to approximately mid-G_1 and decline prior to entrance into the DNA synthetic phase [15, 18, 33]. Cell cycle phase-associated transcriptional controls, thus, appear to be superimposed on this growth state-dependent program of PAI-1 gene regulation following exit from G_0 [15]. While the specific signals for PAI-1 promoter activation during the G_0-G_1 transition are unknown, the E box at nucleotides −165 to −160 in the PAI-1 5′ flanking genomic region is footprinted in constitutively growing HTC cells [29] and, at least in EC-1 cells, one such binding protein is USF-1.

First described as bHLHZ *trans*-activators of adenovirus major-late promoter expression via an upstream E box motif (CACGTG) [34, 35], the bHLHZ proteins USF-1 and USF-2 influence expression of several cellular genes (e.g., γ-fibrinogen, class 1 alcohol dehydrogenase, cyclin D1, p53, and human PAI-1) [36]. These factors appear to have a complex mode of action and likely facilitate TFII-D binding to the TATA box [34, 37]. While USF may also stimulate Inr-dependent transcription by stabilizing the TFII-I preinitiation complex [38, 39], the MYC protein negatively influences Inr-mediated transcriptional responses by sequestering TFII-I [40]. Depending upon the precise promoter configuration (TATA box, Inr, and/or an E box motif), the availability of specific bHLHZ transcription factors with E box recognition domains (e.g., USF and MYC), and elements composing the preinitiation complex, therefore, the effects on PAI-1 transcription

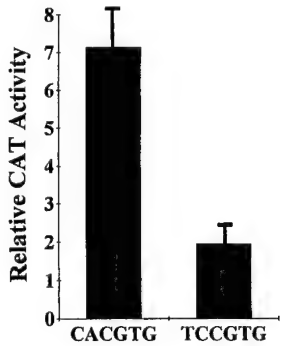

FIG. 8. Functional analysis of PAI-1 promoter fragments containing a wild-type or mutant E box at nucleotides −160 to −165. The PAI-1 consensus E box motif (CACGTG) in rPAI-CAT(−764) was mutated to TCCGTG. Following transient transfection of wild-type and mutant expression vectors into EC-1 cells, CAT reporter activity in growing cells was assessed by thin-layer chromatography and normalized to transfection efficiency. CAT levels driven by the wild-type E box promoter were significantly greater (3.6-fold) than that derived from the plasmid bearing a mutant E box ($P < 0.001$; two-tailed Student t test).

may be different (activation vs repression) [41–43]. In fact, while USF is traditionally thought of as a stimulatory transcription factor, in several systems USF-1 and 2 have repressive or negative regulatory effects [44, 45]. Such inhibition appears due to direct interference with the cellular activator TFE3 [45]. Indeed, these data are consistent with the suggestion that USF does not have an efficient *trans*-activating domain and that displacement of a strong activator by USF downregulates gene expression [45]. Since USF-1 effectively converts the transcription factor VP16 from a repressor into an activator, E box occupancy by USF may also facilitate the recruitment of other transcriptional regulatory proteins to the site in addition to functioning independently to *trans*-activate. Clearly, such effects may be inhibitory as well as stimulatory [46] and appear to involve direct protein-protein interactions (e.g., sequestration of Fra1 by USF resulting in transcriptional repression; formation of USF-2/Fos dimers [46, 47]). The changing dynamics of such interactions at the USF-1-bound PAI-1 E box may well influence the growth state-dependent kinetics of PAI-1 expression in serum-stimulated EC-1 cells.

USF dimers as well as MYC/MAX heterodimers recognize the same intronic E box motif within the p53 gene [48]. Supershift and immunodepletion studies suggest, however, that MYC may not be part of the protein complex bound to the PAI-1 E box, at least in constitutively growing cells. Mobility-shift data additionally suggest that the PAI-1 E box is not occupied by USF-1-containing complexes, or USF-1 is not a prominent E box-binding factor, in quiescent cells. The E box site is bound by USF-1, however, at late stages of proliferative activation (i.e., at 20 h postserum addition) as well as in exponentially growing cells. At 20 h after initial re-entry of previously quiescent cells into the division cycle, as well as in asynchronously proliferating cultures, the levels of PAI-1 gene transcription and steady-state mRNA abundance are low relative to 4 h FBS-stimulated cells. When viewed in the specific context of serum-mediated cell growth activation, thus, USF-1 may function as a modulator of PAI-1 expression via the E box motif rather than to upregulate expression in response to serum stimulation. Identification of USF-1 as an E box-bound species does not resolve this issue as overexpression of USF-1 or USF-2 as homodimers effectively repressed rRNA transcription in CHO cells whereas USF-1/USF-2 heterodimeric complexes served to *trans*-activate [49]. Clarification of E box involvement in transcriptional regulation of the PAI-1 gene during the early stages of growth stimulation (i.e., during the $G_0 \rightarrow G_1$ transition), therefore, will necessitate identification of additional E box-binding protein species in quiescent and early (4 h) FBS-stimulated cells. The E box is clearly involved in the regulation of PAI-1 promoter function in growing EC-1 cells, however, and the same mutation that effectively reduces (by 74%) CAT reporter activity in cycling populations abrogates complex formation between USF-1 and PAI-1 E box-specific DNA probes.

Supported by NIH Grants DK46272 (P.J.H.), CA22729 (T.D.G.), and AG12690 (C.B.) and U.S. Army Grant DAMD17-98-1-8015 (P.J.H.).

REFERENCES

1. Almendral, J., Sommer, D., MacDonald-Bravo, H., Burckhordt, J., Perera, J., and Bravo, R. (1988). Complexity of the early genetic response to growth factors in mouse fibroblasts. *Mol. Cell. Biol.* **8,** 2140–2148.
2. Ryseck, R., MacDonald-Bravo, H., Zerial, M., and Bravo, R. (1989). Coordinate induction of fibronectin, fibronectin receptor, tropomyosin and actin genes in serum stimulated fibroblasts. *Exp. Cell Res.* **180,** 537–545.
3. Herschman, H. R. (1991). Primary response genes induced by growth factors and tumor promoters. *Annu. Rev. Biochem.* **60,** 281–319.
4. Muller, R., Mumburg, D., and Lucibello, F. C. (1993). Signals and genes in the control of cell-cycle progression. *Biochim. Biophys. Acta* **1155,** 151–179.
5. Wick, M., Burger, C., Brusselbach, S., Lucibello, F., and Muller, R. (1994). A novel member of human tissue inhibitor of metalloproteinases (TIMP) gene family is regulated during G1 progression, mitogenic stimulation, differentiation and senescence. *J. Biol. Chem.* **269,** 18953–18960.
6. Lanahan, A., Williams, J. B., Sanders, L. K., and Nathans, D. (1992). Growth factor induced delayed early response genes. *Mol. Cell. Biol.* **12,** 3919–3929.
7. Lau, L. F., and Nathans, D. (1987). Expression of a set of growth related immediate early genes in BALB/c 3T3 cells: Coordinate regulation with c-*fos* or c-*myc*. *Proc. Natl. Acad. Sci. USA* **84,** 1182–1186.
8. Burger, C., Wick, M., Brusselbach, S., and Muller, R. (1994). Differential induction of metabolic genes after mitogen stimulation and during normal cell cycle progression. *J. Cell Sci.* **107,** 241–252.
9. Kato, G. J., and Dang, C. V. (1992). Function of the c-Myc oncoprotein. *FASEB J.* **6,** 3065–3072.
10. Kovary, K., and Bravo, R. (1991). The *jun* and *fos* families are both required for cell cycle progression in fibroblasts. *Mol. Cell. Biol.* **11,** 4466–4472.
11. Kalousek, M., Trub, T., Schuermann, M., and Klemenz, R. (1994). T1 is a c-Fos and FosB-responsive gene which is induced by growth factors through multiple signal transduction pathways. *J. Biol. Chem.* **269,** 6866–6873.
12. Ryan, M. P., and Higgins, P. J. (1994). Control of p52 gene expression in normal and transformed rat kidney cells: Relationship between p52 induction and actin cytoarchitecture. *In* "Actin: Biophysics, Biochemistry, and Cell Biology" (J. E. Estes, and P. J. Higgins, Eds.), pp. 215–230, Plenum Press, NY.
13. Folkman J., and Moscona, A. (1978). Role of cell shape in growth control. *Nature* **273,** 345–349.
14. Pardee, A. B. (1989). G1 events and regulation of cell proliferation. *Science* **246,** 603–608.
15. Ryan, M. P., Kutz, S. M., and Higgins, P. J. (1996). Complex regulation of plasminogen activator inhibitor type-1 (PAI-1) gene expression by serum and substrate adhesion. *Biochem. J.* **314,** 1041–1046.

16. Iyer, V. R., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., Lee, J. C. F., Trent, J. M., Staudt, L. M., Hudson, J., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P. O. (1999). The transcriptional response of human fibroblasts to serum. *Science* **283,** 83–87.
17. Ryan, M. P., and Higgins, P. J. (1993). Growth state-regulated expression of p52(PAI-1) in normal rat kidney cells. *J. Cell. Physiol.* **155,** 376–384.
18. Kutz, S. M., Nickey, S. A. T., White, L. A., and Higgins, P. J. (1997). Induced PAI-1 mRNA expression and targeted protein accumulation are early G1 events in serum-stimulated rat kidney cells. *J. Cell. Physiol.* **170,** 8–18.
19. Uno, S., Nakamura, M., Seki, T., and Ariga, T. (1997). Induction of tissue-type plasminogen activator (tPA) and type-1 plasminogen activator inhibitor (PAI-1) as early growth responses in rat hepatocytes in primary culture. *Biochem. Biophys. Res. Commun.* **239,** 123–128.
20. Schneiderman, J., Sawdey, M., Craig, H., Thinnes, T., Bordin, G., and Loskutoff, D. J. (1993). Type 1 plasminogen activator inhibitor gene expression following partial hepatectomy. *Am. J. Pathol.* **143,** 753–762.
21. Thornton, A., Bruzdzinski, C. J., Raper, S. E., and Gelehrter, T. D. (1994). Plasminogen activator inhibitor-1 is an immediate early response gene in regenerating rat liver. *Cancer Res.* **54,** 1337–1343.
22. Vaheri, A., Stephens, R. W., Salonen, E. M., Pollanen, J., and Tapiovaara, H. (1990). Plasminogen activation at the cell surface-matrix interface. *Cell Differen. Dev.* **32,** 255–262.
23. Pollanen, J., Ross, W., and Vaheri, A. (1991). Directed plaminogen activation at the surface of normal and malignant cells. *Adv. Cancer Res.* **57,** 273–328.
24. Guadagnao, T. M., and Assoian, R. K. (1991). G1/S control of anchorage-independent growth in the fibroblast cell cycle. *J. Cell. Biol.* **115,** 1419–1425.
25. Ciambrone, G. J., and McKeown-Longo, P. J. (1990). Plasminogen activator inhibitor type 1 stabilizes vitronectin-dependent adhesions in HT-1080 cells. *J. Cell Biol.* **111,** 2183–2195.
26. William, T. M., and Keir, H. M. (1977). Affinity chromatography of thiol-containing purines and ribonucleic acid. *Biochem. J.* **168,** 595–597.
27. Woodford, T., Schlegel, R., and Pardee, A. (1988). Selective isolation of newly synthesized mammalian mRNA after in vivo labeling with 4-thiouridine or 6-thioguanosine. *Anal. Biochem.* **171,** 166–172.
28. Mu, X. C., Staiano-Coico, L., and Higgins, P. J. (1998). Increased transcription and modified growth state-dependent expression of the plasminogen activator inhibitor type-1 gene characterize the senescent phenotype in human diploid fibroblasts. *J. Cell. Physiol.* **174,** 90–98.
29. Johnson, M., Bruzdzinski, C., Winograd, S. S., and Gelerhter, T. D. (1992). Regulatory sequences and protein-binding sites involved in the expression of the rat plasminogen activator-inhibitor-1 gene. *J. Biol. Chem.* **267,** 12202–12210.
30. Santaren, J., and Bravo, R. (1987). Immediate early induction of a 45k secreted glycoprotein by serum and growth factors in quiescent mouse 3T3 cells. *Exp. Cell Res.* **168,** 494–506.
31. Fisher, F., and Goding, C. R. (1992). Single amino acid substitutions alter helix-loop-helix protein specificity for bases flanking the core CANNTG motif. *EMBO J.* **11,** 4103–4109.
32. Higgins, P. J., and Ryan, M. P. (1989). Biochemical localization of the transformation sensitive 52 kDa (p52) protein to the substratum contact regions of cultured rat fibroblasts. *Biochem. J.* **257,** 173–182.
33. Mu, X. C., and Higgins, P. J. (1995). Differential growth state-dependent regulation of plaminogen activator inhibitor type-1 expression in senescent IMR-90 human diploid fibroblasts. *J. Cell. Physiol.* **165,** 647–657.
34. Sawadogo, M., and Roeder, R. G. (1985). Interaction of a gene specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell* **43,** 165–175.
35. Lin, Q., Luo, X., and Sawadogo, M. (1994). Archaic structure of the gene encoding transcription factor USF. *J. Biol. Chem.* **269,** 23894–23903.
36. Littlewood, T. D., and Evan, G. E. (1994). "Protein Profile Transcription Factors 2: Helix-Loop-Helix," Vol. 1, Academic Press, NY, p 639–709.
37. Workman, J. L., Roeder, R. G., and Kingston, R. E. (1990). An upstream transcription factor, USF (MLTF), facilitates the formation of preinitiation complexes during *in vitro* chromatin assembly. *EMBO J.* **19,** 1299–1308.
38. Smale, S. T., and Baltimore, D. (1989). The "initiator" as a transcription control element. *Cell* **57,** 103–113.
39. Du, H., Roy, A. L., and Roeder, R. G. (1993). Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. *EMBO J.* **12,** 501–511.
40. Roy, A. L., Carruthers, C., Gutjahr, T., and Roeder, R. G. (1993). Direct role for Myc in transcription initiation mediated by interactions with TFII-I. *Nature* **365,** 359–361.
41. Prendergast, G. C., and Cole, M. D. (1989). Posttranscriptional regulation of cellular gene expression by the c-Myc oncogene. *Mol. Cell. Biol.* **9,** 124–134.
42. Riccio, A., Pedone, P. V., Lund, L. R., Olsen, T., Olsen, H. S., and Andreasen, P. A. (1992). Transforming growth factor β1-responsive element: Closely associated binding sites for USF and CCAAT-binding transcription factor-nuclear factor 1 in the type-1 plasminogen activator inhibitor gene. *Mol. Cell. Biol.* **12,** 1846–1855.
43. Hopewell, R., Li, L., MacGregor, D., Nerlov, C., and Ziff, E. B. (1995). Regulation of the cell proliferation and differentiation by Myc. *J. Cell Sci.* **19,** 85–89.
44. Lun, Y., Sawodogo, M., and Perry, M. (1997). Autoactivation of *Xenopus* MyoD transcription and its inhibition by USF. *Cell Growth Differen.* **8,** 275–282.
45. Carter, R. S., Ordentlich, P., and Kadesch, T. (1997). Selective utilization of basic helix-loop-helix-leucine zipper proteins at the immunoglobulin heavy-chain enhancer. *Mol. Cell. Biol.* **17,** 18–23.
46. Pognonec, P., Boulukos, K. E., Aperlo, C., Fujimoto, M., Ariga, H., Nomoto, A., and Kato, H. (1997). Cross-family interaction between the bHLHZip USF and Bzip Fra1 proteins results in down-regulation of AP-1 activity. *Oncogene* **14,** 2091–2098.
47. Blanar, M., and Rutter, W. (1992). Interaction cloning: Identification of a helix-loop-helix zipper protein that interacts with c-*fos*. *Science* **256,** 1014–1018.
48. Reisman, D., and Rotter, V. (1993). The helix-loop-helix containing transcription factor USF binds to and transactivates the promoter of the p53 tumor suppressor gene. *Nucleic Acids Res.* **21,** 345–350.
49. Ghosh, A. K., Data, P. K., and Jacob, S. T. (1997). The dual role of helix-loop-helix-zipper protein USF in ribosomal RNA gene transcription *in vivo*. *Oncogene* **14,** 589–594.

Received May 3, 2000
Revised version received July 17, 2000

JOURNAL OF CELLULAR PHYSIOLOGY 182:269–280 (2000)

PAI-1 Gene Expression Is Regionally Induced in Wounded Epithelial Cell Monolayers and Required for Injury Repair

KIRWIN M. PROVIDENCE,[1] STACIE M. KUTZ,[1] LISA STAIANO-COICO,[2] AND PAUL J. HIGGINS[1]*

[1]*Cell & Molecular Biology Program, Albany Medical College, Albany, New York*
[2]*Department of Surgery, Weill Medical College of Cornell University, New York, New York*

Induced expression of plasminogen activator inhibitor type-1 (PAI-1), a major negative regulator of pericellular plasmin generation, accompanies wound repair in vitro and in vivo. Since transcriptional control of the PAI-1 gene is superimposed on a growth state–dependent program of cell activation (Kutz et al., 1997, J Cell Physiol 170:8–18), it was important to define potentially functional relationships between PAI-1 synthesis and subpopulations of cells that emerge during the process of injury repair in T2 renal epithelial cells. Specific cohorts of migratory and proliferating cells induced in response to monolayer trauma were spatially as well as temporally distinct. Migrating cells did not divide in the initial 12 to 20 h postinjury. After 24 h, S-phase cells were generally restricted to a region 1 to 2 mm from, and parallel to, the wound edge. Proliferation of wound bed cells occurred subsequent to wound closure, whereas the distal contact-inhibited monolayer remained generally quiescent. Hydroxyurea blockade indicated, however, that proliferation (most likely of cells immediately behind the motile "tongue") was necessary for maintenance of cell-to-cell cohesiveness in the advancing front, although the ability to migrate was independent of proliferation. PAI-1 mRNA expression was rapidly up-regulated in response to wounding with inductive kinetics approximating that of serum-stimulated cultures. Differential harvesting of T2 cell subpopulations, based on proximity to the injury site, prior to Northern assessments of PAI-1 mRNA abundance indicated that PAI-1 transcripts were restricted to cells immediately bordering the wound or actively migrating and not expressed by cells in the distal contact-inhibited monolayer regions. Such cell location–specific distribution of PAI-1-producing cells was confirmed by immunocytochemistry. PAI-1 synthesis in cells that locomoted into the wound field continued until injury closure. Down-regulation of PAI-1 synthesis and matrix deposition in renal epithelial cells, stably transfected with a PAI-1 antisense expression vector, significantly impaired wound closure. Transfection of the wound repair–deficient R/A epithelial line with a sense PAI-1 expression construct restored both approximately normal levels of PAI-1 synthesis and repair ability. These data indicate that PAI-1 induction is an early event in creation of the wound-activated phenotype and appears to participate in the regulation of renal epithelial cell motility during in vitro injury resolution. J. Cell. Physiol. 182:269–280, 2000.

Extracellular matrix (ECM) restructuring following tissue injury is regulated (both temporally and spatially) by the plasmin-based pericellular proteolytic cascade and by members of the metalloproteinase family (Dano et al., 1985; Laiho and Keski-Oja, 1989; Pilcher et al., 1997; Pollanen et al., 1991). Recent in situ and genetic analyses of the repair process suggest that the migratory, proliferative, and ECM remodeling stages of in vivo wound healing are dependent, to a significant extent, on plasminogen activation (i.e., the conversion of plasminogen to the active broad-spectrum protease plasmin by urokinase plasminogen activator [uPA]) (Pollanen et al., 1987, 1991; Schafer et al., 1994; Okada et al., 1995; Pappot et al., 1995; Jensen and Lavker, 1996; Romer et al., 1996; Carmeliet et al., 1997; Hasenstab et al., 1997; Carmeliet and Collen,

Contract grant sponsor: NIH; Contract grant numbers: DK46272 and GM4246; Contract grant sponsor: Department of the Army; Contract grant number: DAMD17-98-1-8015.

*Correspondence to: Dr. Paul J. Higgins, Cell & Molecular Biology Program (Mailcode-165), Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208.
E-mail: phiggins@ccgateway.amc.edu

Received 30 June 1999; Accepted 10 September 1999

1998). Studies in mice genetically engineered to be deficient in one or more elements in the plasmin activation cascade, for example, have confirmed the importance of uPA and plasmin in cell migration at specific injury sites (e.g., Romer et al., 1996; Carmeliet et al., 1997). Plasminogen activator inhibitor type-1 (PAI-1) functions in this process to negatively regulate plasmin generation by complexing with and inhibiting the catalytic activity of free as well as receptor-bound uPA (e.g., Ellis et al., 1990; Laiho and Keski-Oja, 1989) modulating, thereby, uPA-dependent motility in vivo (Carmeliet and Collen, 1995; Carmeliet et al., 1997). Indeed, it appears that various aspects of an efficient response to trauma, including the growth factor–dependent recruitment of endothelial cells into the wound field (Sato and Rifkin, 1988), require precise control over both the expression and localization of particular proteases and protease inhibitors (Andreasen et al., 1997; Pepper et al., 1993).

Cell type–specific synthesis and subcellular targeting of PAI-1 and uPA appear to be important considerations in the modulation of the pericellular proteolytic balance. Following injury, uPA and PAI-1 are initially produced by cells immediately adjacent to the wound edge in vivo as well as in vitro (Romer et al., 1991; Pawar et al., 1995; Reidy et al., 1995; Staiano-Coico et al., 1996; Carmeliet et al., 1997), where PAI-1 is likely stabilized in an active conformation with ECM-associated vitronectin (Declerck et al., 1988; Mimuro and Loskutoff, 1989a,b; Seiffert et al., 1990; Vassalli et al., 1991). Temporal changes in the expression, focalization, and/or relative activity levels of this protease/inhibitor pair may influence cell migration either as a direct consequence of ECM barrier proteolysis or by modulating cellular adhesive interactions with the ECM (Ciambrone and McKeown-Longo, 1990; Blasi, 1996, 1997; Jensen and Lavker, 1996; Carmeliet et al., 1997). Furthermore, recent findings indicate that PAI-1, specific integrins, and uPA function coordinately to influence adhesive events important in the control of cell movement (Carmeliet and Collen, 1995, 1996; Stefansson and Lawrence, 1996; Blasi, 1997; Waltz et al., 1997).

Proliferation is also an essential, albeit presumed independent, component in the closure of epithelial monolayer wounds (Pawar et al., 1995). The exact relationship between regenerative growth and trauma repair, however, remains to be defined (Jensen and Lavker, 1996; Zahm et al., 1997). Within the setting of induced cellular proliferation and migration, stimulated expression of PAI-1 appears associated with both processes (e.g., Bade and Feindler, 1988; Pepper et al., 1992; Thornton et al., 1994; Ryan et al., 1996; Kutz et al., 1997). Transcriptional regulation of the PAI-1 gene, moreover, is superimposed on a growth state–dependent program, which culminates in a proliferative response (Ryan et al., 1996; Kutz et al., 1997). Kinetic assessments indicate that PAI-1 transcription and mRNA expression, similar to that of uPA (Grimaldi et al., 1986), occur early and in immediate-early response (IER) fashion on addition of serum to quiescent cells (Ryan and Higgins, 1993; Ryan et al., 1996; Uno et al., 1997), thereby mimicking regenerative events in vivo (Schneiderman et al., 1993; Thornton et al., 1994). The PAI-1 gene, however, exhibits a complex mode of regulation on entry of G_0-arrested cells into a cycling G_1 condition (Ryan et al., 1996). Serum-induced PAI-1 transcription is maximal in mid-G_1 and declines abruptly prior to the onset of DNA synthesis (White et al., 1999). The amplitude as well as maintenance of expression through mid-G_1 phase is anchorage responsive and this latter adhesion-dependent requirement, unlike initial induction, involves secondary (i.e., protein synthesis–dependent) transcriptional-level events (Ryan et al., 1996). These data provided for a model of PAI-1 gene control in serum-stimulated cells, which incorporates both IER and secondary regulatory influences within an "activated" G_1 state (Kutz et al., 1997; Mu et al., 1998). Such fine control over the kinetics of PAI-1 expression appears to be one modulating aspect in the complexity of G_1 progression. In this regard, PAI-1 may regulate cell-to-substrate adhesion (a necessary prerequisite for G_1/S transition [Guadagno and Assoian, 1991; Guadagno et al., 1993]) or cell shape (and shape-dependent metabolic pathways [e.g., Higgins et al., 1994; Hawks and Higgins, 1998]) by directly influencing the immediate pericellular proteolytic microenvironment (Laiho and Keski-Oja, 1989). PAI-1-dependent ECM stabilization, moreover, may indirectly facilitate the formation of cell-ECM interactions necessary for cellular adhesion and/or migration (e.g., Planus et al., 1997).

Such in vitro observations, in fact, do have in vivo correlates. The morphology of the flattened regenerating renal epithelium, for example, is dramatically different from the normally quiescent highly polarized tubular cell (e.g., Wallin et al., 1992) and PAI-1 expression occurs specifically in regenerating proximal tubular cells following ischemia-reperfusion renal injury (Basile et al., 1998), suggesting a functional role in tissue repair. Clearly, the association between the activated phenotype and targeted accumulation of PAI-1 in the cellular undersurface in close proximity to newly formed focal adhesions is consistent with this function (Kutz et al., 1997). The time course of induced PAI-1 expression and the involvement of the PAI-1-synthesizing cellular cohort in the response to injury, however, are poorly understood. It is important, therefore, to clarify the kinetics of PAI-1 expression with regard to the emergence of specific cell subpopulations (i.e., migratory, proliferating) involved in repair growth and to assess the role of this protease inhibitor in the regenerative process.

MATERIALS AND METHODS

Cell lines and in vitro repair assay

The independent clonal isolates (EC-1 and T2), derived from an early-passage culture of normal rat kidney (NRK) epithelial cells (Ryan and Higgins, 1993), were maintained in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) (DMEM/10). Cells were grown to confluency and the media changed to fresh DMEM/10 for maintenance in a postconfluent condition for an additional 3 days. Alternatively, confluent cultures were maintained in serum-free DMEM for 3 days to initiate a contact-inhibited/serum-deprivation state of growth arrest. Wounds were created by pushing the narrow end of a sterile P1000 plastic pipette tip (Continental Laboratory Products, San Diego, CA) through the monolayer. Cultures were incubated

in the existing media for times indicated in the text. Wound closure was assessed by time-lapse photomicroscopy and injury repair rates calculated, as a function of time, from measurements made utilizing an inverted microscope fitted with a calibrated ocular grid.

Growth "activation" of cells arrested in serum-deficient medium

The growth medium in low-density 150-mm dish cultures was aspirated, the cells rinsed twice in Hanks' balanced salt solution (HBSS) (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.14 M NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose), and serum-free DMEM added (which initiates a rapid G_0 arrest of NRK cells; Kutz et al., 1997). After 3 days, cultures were left untreated or exposed to actinomycin D (5 μg/ml), puromycin (20 and 100 μg/ml), or genistein (50 and 100 μM) for 30 min prior to, and during, stimulation with FBS (added directly to the quiescence maintenance medium to a final concentration of 20%). Cells were harvested for analysis of PAI-1 transcripts (described later) at times indicated in the text.

RNA analysis of cell subpopulations

Cells immediately adjacent to the wound (termed *edge-isolates*) were harvested by pushing the blunt (wide) end of a P1000 plastic pipette tip along the existing wound tract, displacing cells directly at, and 5 mm from, the wound edge; such scrape-released cells were subsequently collected by centrifugation at 1400 × g. Cells located between 10 and 40 mm from the wound border (i.e., in the intact monolayer regions) were collected in the same manner and termed *monolayer-isolates.* In some cases, the entire culture population was harvested with a cell scraper (*total dish–isolates*). Cellular RNA was extracted (Kutz et al., 1997) and denatured by incubation at 55°C for 15 min in 1X MOPS, 6.5% formaldehyde, and 50% formamide prior to electrophoresis (10 μg RNA/lane) on agarose/formaldehyde gels (1.2% agarose, 1.1% formaldehyde, 1X MOPS, pH 8.0) for 3 h at 70 V in 1X MOPS. Fractionated RNA was transferred to positively charged nylon membranes via downward capillary action using the turboblotter system (Schleicher & Schuell, Keene, NH) and UV crosslinked. Hybridization with [^{32}P]-dCTP-labeled cDNA probes to rat PAI-1 and mouse A50 was as described (Ryan et al., 1996). Blots were exposed to X-OMAT AR-5 film (Kodak, Rochester, NY) or analyzed with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA) for visualization and quantitation of mRNA species.

Immunocytochemistry

Cultures were washed twice in Ca^{2+}/Mg^{2+} free phosphate–buffered saline (CMF-PBS; 2.7 mM KCl, 1.2 mM KH_2PO_4, 0.14 M NaCl, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$) and fixed for 10 min at room temperature in 10% formalin/CMF-PBS. Following permeabilization with cold (4°C) 0.5% Triton X-100/CMF-PBS for 10 min at 4°C, cells were washed three times (5 min each) with CMF-PBS prior to incubation (3 h at room temperature) with rabbit antibodies to rat PAI-1 (Kutz et al., 1997) diluted in BSA (3 mg/ml)/CMF-PBS. After three CMF-PBS washes, cells were incubated with fluorescein-conjugated goat anti-rabbit IgG (Chemicon, Temecula, CA; diluted 1:20 in BSA/CMF-PBS) for 30 min at 37°C, washed, and coverslips mounted with 100 mM *n*-propygalate in 50% glycerol/CMF-PBS. Where indicated, 4 μM monensin (Sigma, St. Louis, MO) was added to wounded monolayers prior to fixation. For identification of DNA-synthesizing cells, 5-bromo-2′-deoxyuridine (BrdU) (200 μmol/l final concentration) was added to cultures at time of wounding. The number and location of cells that incorporated the analogue (S-phase cells) were assessed by indirect immunofluorescence, using monoclonal antibodies to BrdU (Mu et al., 1998).

Construction and transfection of sense and antisense PAI-1 expression vector

pBluescript, containing a full-length rat PAI-1 cDNA (Higgins and Ryan, 1992), was digested with *Eco*RI and *Hind*III to generate a 2.6-kb insert (representing nucleotides −118 to +2572 relative to the start site of transcription). Agarose gel–purified DNA was blunt-ended with Klenow fragment/dNTPs using a fill-in reaction. *Not*I linkers were ligated, the fragments digested with *Not*I, and purified by agarose gel electrophoresis. Flanked inserts were ligated to *Not*I-digested calf intestinal phosphatase-treated gel-purified Rc/CMV expression vector DNA and subsequently transformed into competent INVαF′ *E. coli*. Plasmid DNA was isolated from ampicillin-resistant colonies; restriction endonuclease digestion and Southern blot analysis, using a 726-bp *Pst*I/*Apa*I-digested cDNA fragment as a probe, confirmed sense (Rc/CMVPAI) and antisense (Rc/CMVIAP) insert orientation (Higgins et al., 1997). Insert template activity was assessed in vitro for both constructs using T7 polymerase to initiate PAI-1 antisense and sense transcripts (confirmed by hybridization analysis and coupled STP3 transcription-translation [Novagen]/Western blotting, respectively). Cells were transfected with 20 μg of plasmid DNA using the calcium phosphate technique (Higgins et al., 1991). Stable transfectants were selected with G418 (150 μg/ml), and resistant clones were isolated and expanded in growth medium containing G418. Since T2 cells were highly resistant to G418, stable antisense-expressing clones were derived using the EC-1 line.

Metabolic labeling, cell extraction, and gel electrophoresis

Cells were washed with HBSS, then labeled in serum-/methionine-free RPMI 1640 medium containing 50 μCi [^{35}S]methionine/ml (specific activity = 1100 Ci/mmol) (Ryan and Higgins, 1993). The conditioned labeling medium was aspirated and monolayers washed with CMF-PBS prior to extraction with 0.2% (w/v) saponin in CMF-PBS to isolate cell-substratum contact regions and associated undersurface proteins (Higgins et al., 1990, 1991). Saponin-resistant (SAP fraction) residues were scraped into sample buffer (50 mM Tris/HCl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol) and boiled. For electrophoresis, 25,000 cpm trichloroacetic acid–insoluble SAP-fraction protein were separated on SDS/9% acrylamide slab gels (Ryan and Higgins, 1988). Labeled protein bands were visualized by fluorography and quantified by computerized densitometry (Smith et al., 1992). Identification

TABLE 1. Kinetics of wound closure in scrape-injured T2 cell monolayers

Hours postwounding[1]	Wound width[2]	% Injury repair	Closure rate[3]
0	9.68 ± 0.20	0	—
2	9.21 ± 0.22	4.8	2.4
4	8.66 ± 0.29	10.5	2.6
6	8.35 ± 0.33	13.7	2.3
9	7.23 ± 0.39	25.3	2.8
12	6.32 ± 0.43	34.0	2.8
21	3.97 ± 0.51	58.9	2.8
26	2.18 ± 0.58	77.0	2.9
30	1.62 ± 0.45	83.3	2.8
36	0	100	—

[1]A contact-inhibited monolayer of T2 cells was scrape-wounded and the extent of injury repair (measured by a calibrated ocular grid and expressed as percent closure) plotted as a function of time postinjury.
[2]Width of the remaining unhealed region was measured in arbitrary units with a calibrated ocular grid. Data represent the mean ± SD of 10 individual assessments.
[3]Closure rate $= \frac{\text{mean \% repair}}{\text{time (h) postwounding}}$

of the rat PAI-1 protein in one-dimensional electrophoretic separations utilized criteria described previously (Higgins et al., 1989, 1990) as well as by immunochemical reactivity with PAI-1-specific antibodies (Higgins et al., 1990).

RESULTS

Kinetics of the repair response

Cellular movement into the wound "bed" occurred relatively quickly (i.e., within 1 h after monolayer scraping). Trauma site closure proceeded at a constant rate and was complete by 30 to 36 h after injury in this model (Table 1). Time-lapse videomicroscopy and examination of fixed, acridine orange-stained, T2 cell cultures confirmed that relatively close cell-to-cell contact in the migrating tongues, as well as within the distal monolayer regions, was maintained throughout the repair process (i.e., solitary migratory cells did not enter the denuded zone; see later discussion). An absence of mitotic cells in the migratory cohort was also apparent in the time-lapse assessments, suggesting that the mobile population does not divide (at least during the initial 12 to 20 h after wounding). Mitotic cells were evident, however, in regions of the monolayer approximately 1 to 2 mm from, and parallel to, the edge of the original scrape injury (at 24 h after wounding) and within the confines of the initial injury site (only after the opposing fronts made contact) but not in the distal monolayer.

This regional compartmentalization of T2 cells with differing proliferative kinetics, moreover, suggests that a relationship exists between entry into the division cycle and distance from the wound. To assess this possibility, the culture medium was supplemented with 200 μmol/l BrdU at the time of scraping specifically to identify cells that enter S phase in response to injury. This quantitative approach, when applied over the entire time course of monolayer repair, confirmed that (1) confluent T2 cells are, in fact, contact inhibited; (2) 24 h postinjury, the majority of S-phase cells concentrate in a region approximately 1 to 2 mm from, and parallel to, the wound edge and (to a lesser extent) in some leading edge cells; and (3) proliferation of cells that had entered

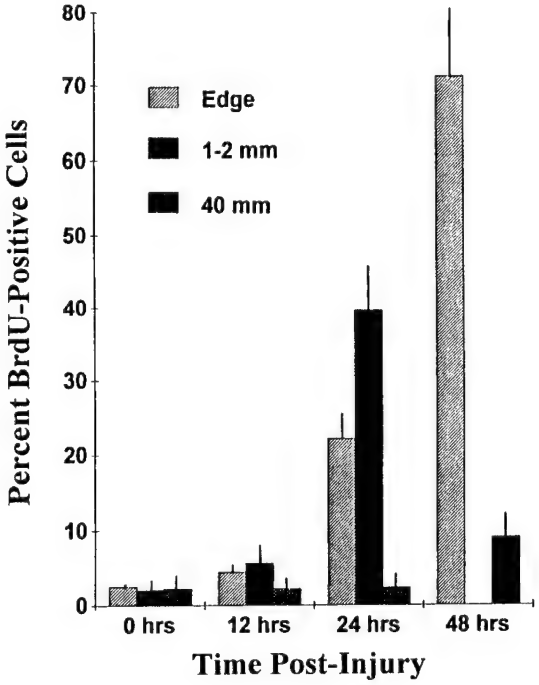

Fig. 1. T2 cells synthesize DNA as a function of their spatial relationship to the wound site. The fraction of S-phase cells is low in uninjured monolayers or in scraped cultures at early times (i.e., ≤12 h) postinjury. By 24 h after wounding, most proliferating cells (as determined by immunocytochemical detection of BrdU incorporation) occur within the first 1 to 2 mm from, and parallel to, the wound edge, although S-phase cells also begin to appear at the leading edge. Cells in the distal monolayer (i.e., 40 mm from the injury site) remain generally quiescent throughout the repair process. The fraction of DNA-synthesizing cells in the distal regions increases only slightly (i.e., comprising <10% of the total population) after complete closure (by approximately 36 h). The healed wound bed population (i.e., "Edge" cells at the 48-h time point) proliferate vigorously in the postclosure period. Percent S-phase (BrdU-positive) cells was determined by counting 10 random fields representative of each of the indicated culture areas for three independent experiments; data plotted represent the mean ± SD of such assessments.

the wound bed occurs subsequent to scrape closure (Fig. 1). The presence or absence of conditioned serum had no significant effect on repair regional kinetics (i.e., the rate of closure differed by only 10 to 15% for cultures wounded in serum-containing as compared to serum-free medium).

To ascertain whether migration alone is sufficient to achieve wound closure, T2 monolayers were scrape-injured in the presence of 0.4 mM hydroxyurea (HU) to inhibit ribonucleoside diphosphate reductase activity (Krakoff et al., 1968; Wang et al., 1997). Preliminary experiments indicated that this concentration of HU effectively inhibited serum-induced DNA synthesis in NRK cells. HU-treated T2 cultures exhibited the same initial morphologic response to wounding as control populations (i.e., membrane ruffling and cell spreading occurred within 30 min of injury followed by migration into the denuded area). By 12 h postinjury, however, the integrity of the migrating sheet was lost in HU-treated cells; after 24 h (a time point that correlated with a significant increase in the fraction of BrdU-positive cells in control cultures; e.g., Fig. 1), there was a complete HU-associated loss of cell-to-cell contact at

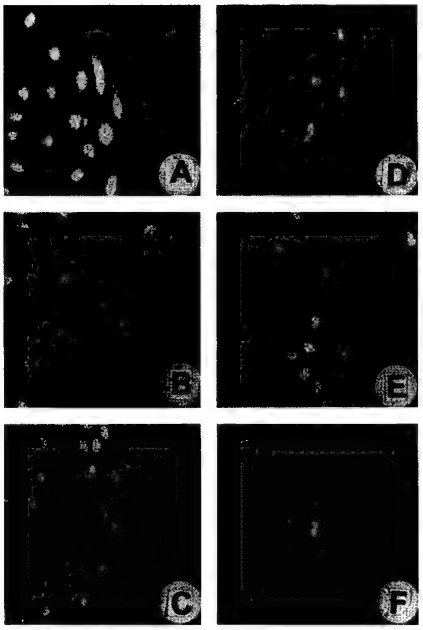

Fig. 2. Effects of hydroxyurea (HU) on the repair response by T2 cells. In control wounded monolayers, the migratory tongue advanced as a generally uniform sheet of closely juxtaposed cells (**A, B, C** = 6, 12, 24 h, respectively, after scraping). Such cohesion was lost in HU-treated cultures (**D, E, F** = 6, 12, 24 h, respectively, postinjury). HU-associated loss of cell-to-cell contact was evident within 12 h (E) and clearly obvious by 24 h (F) postwounding; cell morphology was unaffected by HU. In each panel, the direction of migration is from left to right into the denuded zone. For acridine orange histochemistry, cells were permeabilized in 0.08 N HCl, 0.15 M NaCl, and 0.1% Triton X-100 for 30 sec, then incubated in staining buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 6.0, 1 mM disodium EDTA, 0.15 M NaCl, 6 μg acridine orange/ml). UV light microscopy, ×50.

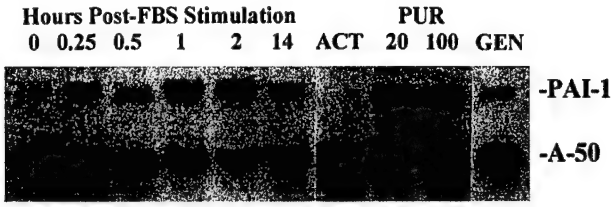

Fig. 3. Time course and metabolic characteristics of serum-induced PAI-1 gene expression. Quiescent T2 cell cultures were stimulated by direct addition of serum (to a final concentration of 20%); control cultures were maintained in serum-free DMEM (time 0). RNA was isolated at the times indicated post-FBS addition. For assessment of the metabolic requirements of serum-induced PAI-1 expression, cells were pretreated with actinomycin D (ACT; 5 μg/ml), puromycin (PUR; 20 or 100 μg/ml), or genistein (GEN; 100 μM) prior to addition of FBS; RNA was extracted from inhibitor-treated cultures 4 h after serum stimulation. Northern blots were hybridized with ^{32}P-labeled PAI-1 and A-50 cDNA probes simultaneously. PAI-1 transcriptional activation in response to serum had IER characteristics and was genistein-sensitive. Identical results were obtained in four different experiments.

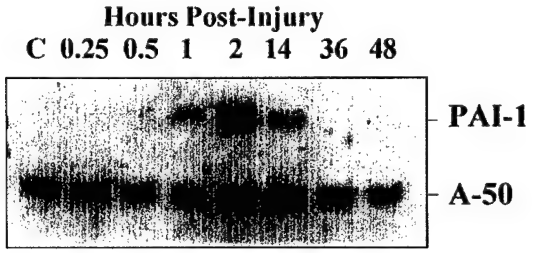

Fig. 4. Induction of PAI-1 in mRNA response to wounding. Confluent T2 monolayers were scrape-wounded and RNA from total dish-isolates extracted at the times indicated. Northern blots were hybridized with ^{32}P-labeled PAI-1 and A-50 cDNA probes simultaneously. PAI-1 transcripts were evident as early as 30 min to 1 h after injury, expression was maximal at 2 h, and declined to levels comparable to quiescent controls by 36 h. C = quiescent nonwounded T2 cells. Blot shown is representative of three individual experiments.

the leading edge (Fig. 2). Migratory activity, however, appeared unaffected by HU, since cells from opposing, albeit noncohesive, "fronts" made contact within the same time frame (i.e., 30 to 36 h) as in untreated populations. Migration, therefore, occurs independently of proliferation, although proliferation (most likely of cells immediately behind the mobile tongue) is necessary for maintenance of monolayer integrity in the zone of repair.

Characteristics of induced PAI-1 expression in wound-"activated" cultures

The temporally and spatially distinct phases of migration and proliferation are associated with the wound-"activated" phenotype (Figs. 1 and 2). It was, therefore, important to assess whether induced PAI-1 expression was an element in this repair response and, if so, to determine both the time course of PAI-1 transcript synthesis and in situ distribution of PAI-1 expressing cells relative to the site of injury. Similar to serum-stimulation of quiescent NRK cell cultures (used for a kinetic comparison; e.g., Fig. 3), wounding-induced PAI-1 mRNA transcripts are first detectable in total dish–isolates of T2 cells within 30 min to 1 h after monolayer scraping (Fig. 4). PAI-1 mRNA is maximally expressed between 1 and 2 h posttrauma, declining dramatically by 14 h (Fig. 4) and closely approximating the changing transcript abundance typical of serum-stimulated cultures (Fig. 3). To ascertain specifically which cells within injured cultures express PAI-1, RNA was extracted from individual edge- and monolayer-isolates at various times postscraping. PAI-1 mRNA was rapidly induced (by 2 h) after injury in edge-isolates, declined quickly thereafter, and by 24 h post-trauma PAI-1 mRNA levels in cells at the injury site approximated that of nonwounded controls (Fig. 5). PAI-1 transcript abundance in distal monolayer-isolates, in contrast, did not change over the time course of repair, remaining similar to that of quiescent cultures.

In situ assessments of PAI-1 accumulation confirmed the RNA analysis. Consistent with the Northern blot data (Figs. 4 and 5), contact-inhibited T2 cells expressed only low levels of PAI-1 protein. Continuous migration into the denuded area, combined with the observation that the distal monolayer does not express PAI-1 mRNA (Fig. 5), suggests that PAI-1 protein synthesis may be restricted to leading edge cells and cells proximal to the site of injury (i.e., within the region of the migrating tongue). To address this issue, monensin (a Na^+/K^+ ionophore that interferes with several metabolic functions, including protein secretion but not synthesis [Ledger et al., 1980; Uchida et al., 1980]) was

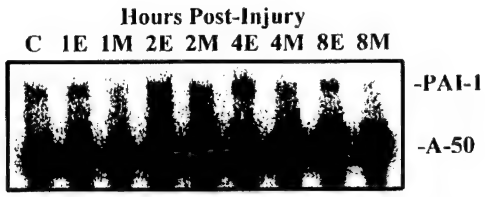

Fig. 5. Regional specificity of induced PAI-1 mRNA expression. Confluent T2 monolayers were wounded and RNA isolated at various times postinjury from cells bordering the wound edge (E = edge-isolates) or in the distal monolayer region (M = monolayer-isolates). Northern blots were hybridized with ^{32}P-labeled PAI-1 and A-50 cDNA probes simultaneously; blot shown is representative of triplicate experiments. PAI-1 transcript expression was restricted to cells at the wound edge. The time course of PAI-1 induction in edge-isolates was similar to that of total dish-isolates (e.g., Fig. 4).

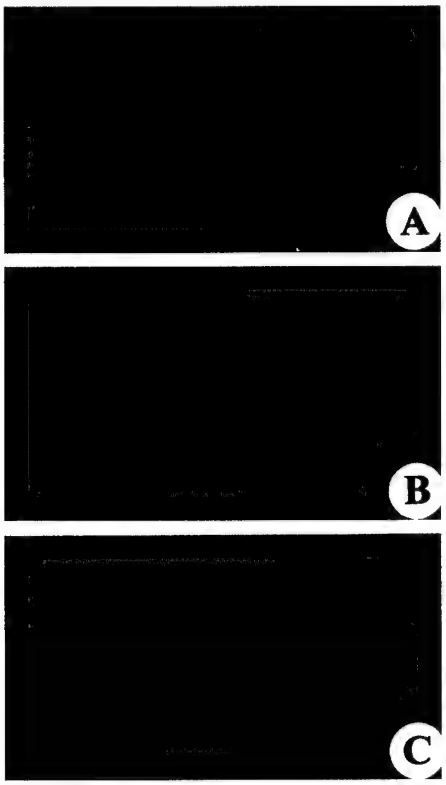

Fig. 6. Relationship between PAI-1 immunoreactive T2 cells and site of injury. Monensin treatment was used to inhibit protein secretion to maximize the likelihood of detection of de novo synthesized PAI-1 protein. In response to wounding, PAI-1 protein specifically localizes to cells adjacent to the wound edge with minimal synthesis by distal monolayer region cells. (**A**) nonwounded monolayer; (**B**) edge, 4 h postinjury; and (**C**) monolayer, 4 h postinjury. Indirect immunofluorescence microscopy, ×185.

added to cultures at the time of wounding. Immunocytochemical evaluation of monensin-treated cultures confirmed the Northern assessments; de novo PAI-1 protein synthesis occurs primarily by cells immediately adjacent to the wound edge (Fig. 6). Coincident with the wound-induced expression of PAI-1 mRNA transcripts (e.g., Fig. 5), and at every time point thereafter, immunoreactive PAI-1 protein was resolved both at the migrating edge and within cells proximal to the closing wound bed. PAI-1 synthesis continues until wound closure with only minimal expression in the distal monolayer.

TABLE 2. Inhibition of wound closure by genistein

Serum[1]	Hours postwounding	Treatment[2]	% Injury repair[3]
None	2	DMSO	4.43 ± 0.62
		50 μM	4.92 ± 0.92
		100 μM	3.61 ± 1.69
None	12	DMSO	35.01 ± 6.37
		50 μM	22.62 ± 3.33
		100 μM	15.62 ± 3.34
None	24	DMSO	76.97 ± 0.83
		50 μM	41.05 ± 6.59
		100 μM	27.67 ± 6.19
10%	2	DMSO	4.72 ± 0.83
		50 μM	4.18 ± 0.48
		100 μM	3.58 ± 0.54
10%	12	DMSO	39.83 ± 2.99
		50 μM	27.66 ± 4.38
		100 μM	20.61 ± 3.24
10%	24	DMSO	92.27 ± 3.41
		50 μM	61.59 ± 5.72
		100 μM	46.89 ± 7.73

[1]Contact-inhibited monolayers (in serum-free or 10% FBS–containing DMEM) of T2 cells were scrape-wounded and the extent of injury repair (measured by a calibrated ocular grid and expressed as percent closure) plotted as a function of time postinjury (as in Table 1).
[2]Cultures were incubated in DMSO (solvent control) or the indicated concentrations of genistein by direct addition to the maintenance medium immediately after wounding.
[3]Data represent the mean ± SEM for three independent experiments.

Consequences of PAI-1 expression manipulation on in vitro wound repair

Location-specific induced expression of PAI-1 mRNA and protein within the wound field (e.g., Figs. 4 to 6) suggested that, if PAI-1 was functionally related to the repair process, inhibition of synthesis by cells at the site of trauma might affect either the time course of injury resolution or the recruitment of cells into the denuded zone. Monensin was not appropriate for this evaluation. Initial studies indicated that this drug did reduce, but not completely inhibit, PAI-1 secretion and accumulation in the cellular undersurface region. Thus, monensin was useful for in situ studies (e.g., Fig. 6) but of limited value for functional assessments. Additional pharmacologic and molecular genetic approaches were designed, therefore, to address this issue. The tyrosine kinase inhibitor genistein was selected as the pharmacologic agent, since cell shape–dependent PAI-1 transcription (Hawks and Higgins, 1989), serum-induced PAI-1 expression (Fig. 3), and cell proliferation/angiogenic responses (Fotsis et al., 1993) are sensitive to genistein treatment. Initial experiments indicated that the time course of PAI-1 mRNA expression by NRK cells bordering the wound margin was similar to that of serum-stimulated cells (Figs. 5 and 6); moreover, PAI-1 induction in response to serum (e.g., Fig. 3) and scrape injury (data not shown) was inhibited by genistein. Genistein-mediated suppression of PAI-1 transcription was evident at concentrations of inhibitor that effectively reduced migration (e.g., Table 2).

A molecular genetic approach was devised, therefore, in which EC-1 cells were stably transfected with an

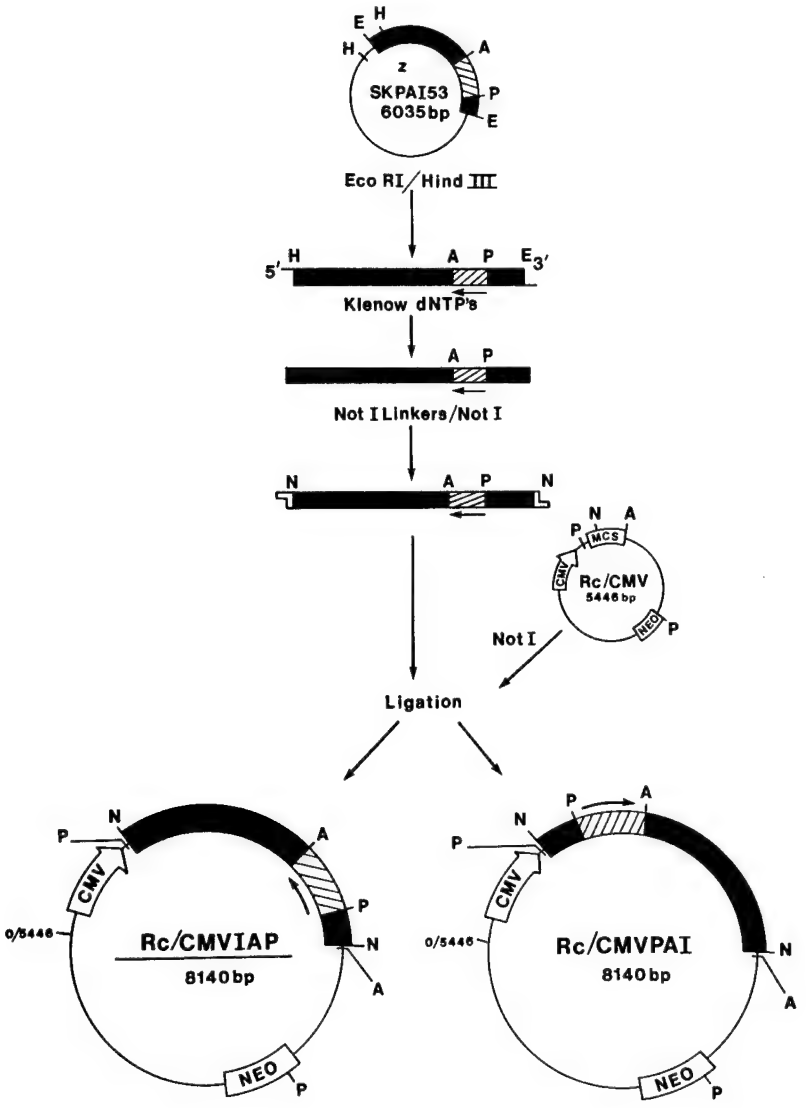

Fig. 7. Construction of sense (PAI) and antisense (IAP) expression vectors. Cloning of PAI-1 cDNA (nucleotides −118 to +2572) into the *Not*I site of the 5.4-kb Rc/CMV vector was as detailed (Materials and Methods). Topography of known restriction sites (P, *Pst*I; N, *Not*I; A, *Apa*I) was used to assess insert orientation.

expression vector (Rc/CMVIAP) bearing a PAI-1 cDNA insert in antisense configuration (Fig. 7) to specifically assess the relationship between wound-induced PAI-1 expression and cell migration. One derived line (Rc/CMVIAP-4HH) was selected for analysis since gel electrophoresis of the SAP fraction protein complement indicated that the 4HH clone was functionally PAI-1 "null" (Fig. 8). Injury site closure by the 4HH derivative was significantly impaired relative to EC-1 parental cells and Rc/CMV transfectants (Fig. 9). Wounds in 4HH monolayers were less than 60% "healed," even at the protracted time of 84 h postscraping, compared to collaterally evaluated controls for which more than 92% repair was typically achieved within the 30 to 36 h window (Fig. 9) and closure complete by 40 h or less. It was not possible to "rescue" the 4HH healing-deficient phenotype by transfection with the Rc/CMVPAI sense expression vector; surviving clones consisted of morphologically aberrant cells and, as such, were unsuited for wounding assays. A previous screen of NRK cells (Higgins et al., 1991), however, resulted in the derivation of one clone (NRK-R/A) that expressed significantly reduced levels of PAI-1 compared to parental EC-1 cells (Fig. 8). NRK-R/A cells also exhibited a marked inability to close monolayer scrape wounds, although this phenotype was not as severe as in the 4HH derivative and may reflect the fact that, unlike the 4HH clone, NRK-R/A cells express at least some PAI-1 (Fig. 8). Transfection of the NRK-R/A line with the Rc/CMVPAI vector restored both approximately normal levels of PAI-1 synthesis and wound repair ability (Fig. 10).

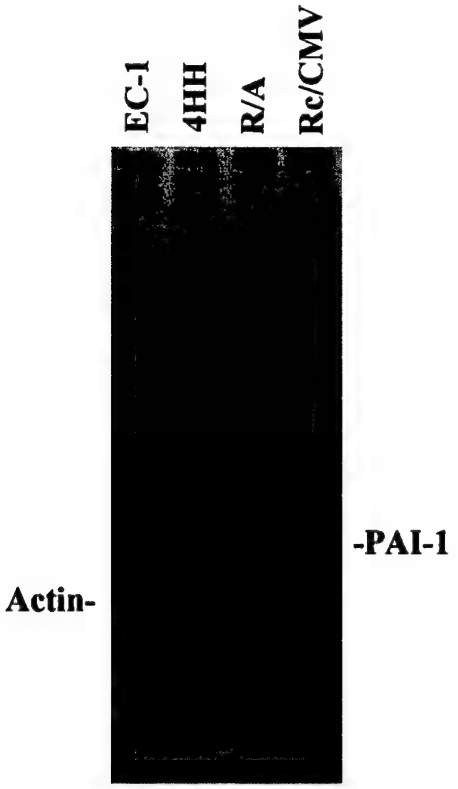

Fig. 8. Relative PAI-1 protein content in the saponin-resistant fraction of control and transfected NRK cells. Cellular SAP fractions (25,000 cpm trichloroacetic acid–insoluble protein) were separated on SDS/9% acrylamide slab gels. Bands corresponding to actin and the 52-kDa PAI-1 protein are indicated. Compared to parental EC-1 cells and insertless vector-transfected controls (Rc/CMV), de novo synthesized PAI-1 protein was virtually undetectable in an antisense PAI-1-expressing derivative (4HH). The R/A isolate (Higgins et al., 1991), which expresses relatively low levels of PAI-1 protein, is also included for comparison. Although actin and PAI-1 are the predominant protein species resolved, it is apparent that the almost complete loss of and reduction in SAP fraction PAI-1 deposition for the 4HH and R/A cell lines, respectively, is highly specific.

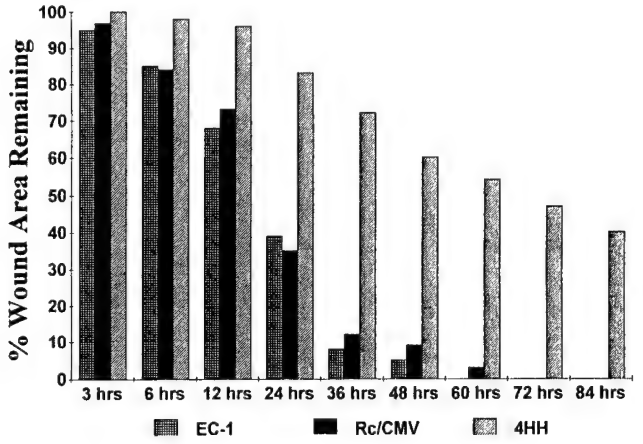

Fig. 9. Kinetics of monolayer scrape wound repair by Rc/CMVIAP-4HH cells compared to parental EC-1 cells and Rc/CMV control transfectants. Confluent monolayers of EC-1, Rc/CMV, and Rc/CMVIAP-4HH cells were maintained in serum-free medium for 3 days, then wounded, and the time course of injury repair assessed as described (Materials and Methods). Whereas the repair rate for EC-1 and Rc/CMV cells was virtually identical, wounds created in monolayers of 4HH cells exhibited little migratory activity and remained open for as long as 84 h postinjury. Data represent the average of duplicate wound repair assays.

DISCUSSION

In vitro migration into scrape-denuded areas is accomplished by the lateral movement of surviving cells across relatively uncomplicated substrates (e.g., Pepper et al., 1987; Sato and Rifkin, 1988; Ando and Jensen, 1996) unlike in vivo transit through fibrin-rich barriers or a provisional wound matrix (Clark et al., 1995; Yamada and Clark, 1995). Despite these considerable physiologic differences, certain commonalites are evident between the two models. Data presented here indicate that cohorts of migrating and proliferating T2 epithelial cells induced in response to monolayer trauma are, at least initially, spatially as well as temporally distinct. Proliferation is required to maintain the integrity of the migrating front, although inhibition of DNA synthesis (with HU) does not affect cell motility. Similar functional compartmentalization occurs as part of the wound repair response in vivo, suggesting that several important phases of injury resolution (regional PAI-1 expression, spatial/temporal distinctions between the motile and proliferative phenotypes) (e.g., Romer et al., 1991; Pawar et al., 1995; Reidy et al., 1995; Staiano-Coico et al., 1996; Zahm et al., 1997) are recapitulated in the T2 epithelial cell system. Migration may be, in fact, a prerequisite to trauma-associated proliferation as the impaired ability of 4HH cells to heal monolayer wounds was reflected in a substantially reduced number of S-phase or mitotic cells in the 1- to 2-mm region distal to the site of injury (data not shown).

Cycles of (leading edge) adhesion and (trailing edge) detachment must be maintained for cells to locomote effectively; such motile cells utilize focal contact–like structures to form transient attachments with the ECM (e.g., Woods and Couchman, 1988). The levels of cell surface uPA and newly synthesized or matrix-bound PAI-1, therefore, may regulate movement by influencing the extent of disruption of integrin-ECM adhesions (Duband et al., 1991; Okedon et al., 1992; McGuire and Alexander, 1993; Deng et al., 1996; Stefansson and Lawrence, 1996; Blasi, 1997; Chapman, 1997). Subcellular targeting of de novo synthesized PAI-1 to the cellular undersurface in close proximity to focal contact sites (Kutz et al., 1997), moreover, can influence uPA-dependent proteolysis and cell attachment, the latter as a consequence of interactions between the uPA/PAI-1/uPA receptor (uPAR) system and vitronectin or between PAI-1 and vitronectin/αv integrins (Loskutoff et al., 1999). Indeed, the uPAR associates with β_2/β_1 integrins, binds to vitronectin (Wei et al., 1994, 1996; Bohuslav et al., 1995; Kanse et al., 1996; Chapman, 1997), and focalizes uPA (Wilcox et al., 1997). Vitronectin absorbed from the medium or newly synthesized (Underwood et al., 1993) at the injury site may serve as the initial "matrix" onto which cells adjacent to the wound can migrate.

Binding of vitronectin to the uPAR, and the formation of uPAR-dependent adhesions, requires uPA (Waltz and Chapman, 1994; Wei et al., 1994). Such

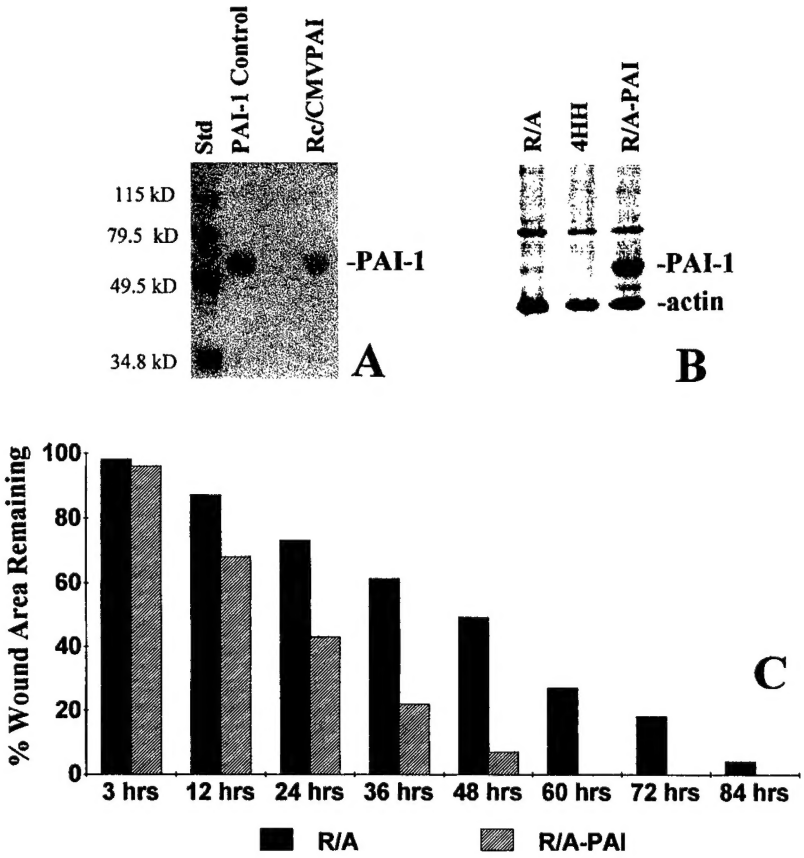

Fig. 10. Effect of vector-driven PAI-1 expression on wound repair by transfectant R/A cells. Coupled in vitro transcription/translation assay confirmed that PAI-1 protein was synthesized using the Rc/CMV-PAI vector as template. When the coupled reaction was complete, the translation products were resolved on a SDS/10% acrylamide gel followed by immunoblotting with PAI-1 antibodies (**A**). Protein standards (Std) and purified PAI-1 protein provided internal controls. Transfection of the Rc/CMVPAI plasmid into R/A cells resulted in derivation of the R/A-PAI line. Gel electrophoresis of the saponin fraction of ^{35}S-methionine-labeled cultures indicated that SAP fraction PAI-1 protein levels expressed by R/A-PAI cells was greater then 11-fold that of the parental R/A line (**B**). The time course of wound closure for R/A-PAI transfectants was significantly reduced relative to R/A cells (**C**), although injury repair by R/A cells (which expressed low levels of PAI-1 compared to EC-1 cells but more PAI-1 than the 4HH derivative; e.g., panel C and Fig. 8) was inhibited relative to EC-1 cells and more effective than 4HH cells (Fig. 9).

uPAR–vitronectin interactions involve the second and third domains of the receptor and are stimulated by pro-PA, uPA, and the isolated A-chain of uPA (Andreasen et al., 1997; Blasi, 1996). PAI-1 binds to the somatomedin B domain of vitronectin, which is the same region that interacts with the uPAR (Deng et al., 1996). The approximately 30-fold greater affinity of PAI-1 for vitronectin, as compared to the affinity of vitronectin for uPAR, suggests a mechanism whereby this SERPIN may effectively dissociate bound vitronectin from uPAR and can detach cells that utilize uPAR as a matrix anchor from a vitronectin substrate (Deng et al., 1996; Loskutoff et al., 1999). Receptor-associated uPA/PAI-1 complexes, moreover, are internalized by endocytosis, which promotes formation of a vacant uPAR on the cell surface by either the internalization of the complex alone or the complex bound to the uPAR followed by receptor recycling (Andreasen et al., 1994, 1997; Blasi, 1996). The available uPAR may bind newly absorbed vitronectin, further supporting adhesive interactions between the cell and the "matrix" to promote lateral migration into the denuded area. Alternatively, PAI-1 may directly inhibit αv integrin-mediated attachment to vitronectin by blocking accessibility to the RGD sequence located proximal to the uPAR binding site (Stefansson and Lawrence, 1996; Loskutoff et al., 1999).

Wound healing appears to be managed by the temporal induction of genes associated with the plasminogen activation cascade, changes in cellular adhesive characteristics, and by the spatial relationship between surviving cells and their distance from the injury site. Enhanced uPA/plasmin activity is important for cell invasion through complex matrices (Meissauer et al., 1991; Kariko et al., 1993; Stahl and Mueller, 1994; Kawada and Umezawa, 1995; Liu et al., 1995). The requirement for uPA activity to transverse over a substratum, however, is less clear and may be cell type dependent. Although both keratinocytes and endothelial cells express increased levels of uPA and PAI-1 in response to monolayer wounding, uPA is apparently not required for keratinocyte locomotion in vitro, whereas occupancy of uPAR by uPA, but not uPA catalytic activity, facilitates wound-responsive endothelial

cell motility (Pepper et al., 1987; Sato and Rifkin, 1988; Okedon et al., 1992; Ando and Jensen, 1996). Present data indicate that PAI-1 expression is an early response to injury and necessary for normal rat kidney–derived epithelial cells to effectively repair monolayer wounds. Since PAI-1 is also critical for invasive growth (Liu et al., 1995; Bajou et al., 1998), the function of PAI-1 in wound repair is likely to be complex. Whether this SERPIN stimulates or inhibits cell motility is likely dependent on both the level and focalization of uPA activity, the composition of the ECM, and the integrin complement of the cell (Kjoller et al., 1997).

These orchestrated events suggest that wounding may be a stimulus that indirectly leads to (genistein-sensitive) signal-transduction events that manage wound healing. In addition to the involvement of local growth factors in cellular reprogramming (e.g., Sato and Rifkin, 1998), uPA binding to its receptor may also transduce signals to the cell interior independent of plasmin generation (Andreasen et al., 1997). uPA–uPAR interactions induce c-*fos* gene expression (Dumler et al., 1994), whereas pro-uPA binding to the u-PAR has been reported to inhibit cell cycle progression of HL-60 cells (Howell et al., 1994), explaining, perhaps, the delay in proliferation by cells of the migratory front. The role of uPA in migration across a denuded zone may be cell type related (Pepper et al., 1987; Sato and Rifkin, 1988; Ando and Jensen, 1996) and appears complicated by differential utilization of uPA/uPAR vs. vitronectin/integrin targets as PAI-1-sensitive motors (e.g., Chapman, 1997; Loskutoff et al., 1999). Regardless of the precise mechanism(s) involved, the present findings, using the complementary approaches of molecular genetic targeting of PAI-1 expression and rescue of a repair-deficient phenotype, strongly suggest that PAI-1 regulates renal epithelial cell motility in response to monolayer wounding. The associated changes in the temporal expression and site-specific localization of PAI-1, moreover, would likely influence the stability of both preexisting and newly formed cell-to-ECM adhesive complexes (Ciambrone and McKeown-Longo, 1990), thereby consistently modulating cellular migratory traits over the time course of injury repair (Blasi, 1993, 1996, 1997).

ACKNOWLEDGMENTS

The help of J. Lehman with videomicroscopy and the assistance of M. Ryan and J. Bollenbocker in the generation and analysis of transfectant clones is gratefully acknowledged.

LITERATURE CITED

Ando Y, Jensen PJ. 1996. Protein kinase C mediates up-regulation of urokinase and its receptor in the migrating keratinocytes of wounded cultures, but urokinase is not required for movement across a substratum in vitro. J Cell Physiol 167:500–511.

Andreasen PA, Scottrup-Jensen L, Kjoller L, Nykaer A, Moestrup SK, Petersen CM, Gliemann J. 1994. Receptor-mediated endocytosis of plasminogen activators and activator/inhibitor complexes. FEBS Lett 338:239–245.

Andreasen PA, Kjoller L, Christensen L, Duffy MJ. 1997. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer 72:1–22.

Bade EG, Feindler S. 1988. Liver epithelial cell migration induced by epidermal growth factor or transforming growth factor alpha is associated with changes in the gene expression of secreted protein. In Vitro Cell Dev Biol 24:149–154.

Bajou K, Noel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D, Foidart JM. 1998. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat Med 4:923–928.

Basile DP, Martin DR, Hammerman MR. 1998. Extracellular matrix-related genes in kidney after ischemic injury: potential role for TGF-β in repair. Am J Physiol 275:F894–F903.

Blasi F. 1993. Urokinase and urokinase receptor: a paracrine/autocrine system regulating cell migration and invasiveness. Bioessays 15:105–111.

Blasi F. 1996. The urokinase receptor and cell migration. Semin Thromb Hemost 22:513–516.

Blasi F. 1997. uPA, uPAR, PAI-1: key intersection of proteolytic, adhesive and chemotactic highways? Immunol Today 18:415–417.

Bohuslav J, Horejsi V, Hansmann C, Stockl J, Weidle UH, Majdic O, Bartke I, Knapp W, Stockinger H. 1995. Urokinase plasminogen activator receptor, beta 2-integrins, and Src-kinases within a single receptor complex of human monocytes. J Exp Med 181:1381–1390.

Carmeliet P, Collen D. 1995. Gene targeting and gene transfer studies of the plasminogen/plasmin system: implications in thrombosis, hemostasis, neointima formation, atherosclerosis. FASEB J 9:934–938.

Carmeliet P, Collen D. 1996. Genetic analysis of the plasminogen and coagulation system in mice. Haemostasis 26(Suppl 4):132–150.

Carmeliet P, Collen D. 1998. Development and disease in proteinase-deficient mice: role of the plasminogen, matrix metalloproteinase and coagulation system. Thromb Res 91:255–285.

Carmeliet P, Moons L, Herbert JM, Crawley J, Lupu F, Lijnen R, Collen D. 1997. Urokinase but not tissue plasminogen activator mediates arterial neointima formation in mice. Circ Res 81:829–839.

Chapman HA. 1997. Plasminogen activators, integrins, the coordinated regulation of cell adhesion and migration. Curr Opin Cell Biol 9:714–724.

Ciambrone GJ, McKeown-Longo PJ. 1990.Plasminogen activator inhibitor type 1 stabilizes vitronectin-dependent adhesions in HT-1080 cells. J Cell Biol 111:2183–2195.

Clark RAF. 1995. Wound repair: overview and general considerations. In: Clark RAF, editor. The molecular and cellular biology of wound repair. New York: Plenum Press. p 3–50.

Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen, LS, Skriver L. 1985. Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 44:139–266.

Declerck PJ, De Mol M, Alessi MC, Baudner S, Paques EP, Preissner KT, Muller-Berghaus G, Collen D. 1988. Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma: identification as a multimeric form of S protein (vitronectin). J Biol Chem 263:15454–15461.

Deng G, Curriden SA, Wang S, Rosenberg S, Loskutoff DJ. 1996. Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? J Cell Biol 134:1563–1571.

Duband JL, Dufour S, Yamada SS, Yamada KM, Thiery JP. 1991. Neural crest cell locomotion induced by antibodies to beta 1 integrins: a tool for studying the roles of substratum molecular avidity and density in migration. J Cell Sci 98:517–532.

Dumler I, Petri T, Schleuning WD. 1994. Induction of c-fos gene expression by urokinase-type plasminogen activator in human ovarian cancer cells. FEBS Lett 343:103–106.

Ellis V, Wun TC, Behrendt N, Ronne E, Dano K. 1990. Inhibition of receptor-bound urokinase by plasminogen-activator inhibitors. J Biol Chem 265:9904–9908.

Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L. 1993. Genistein, a dietary-derived inhibitor of in vitro angiogenesis. Proc Natl Acad Sci USA 90:2690–2694.

Grimaldi G, DiFiore P, Locatelli EK, Falco J, Blasi F. 1986. Modulation of urokinase plasminogen activator gene expression during the transition from quiescent to proliferative state in normal mouse cells. EMBO J 5:855–861.

Guadagno TM, Assoian RK. 1991. G_1/S control of anchorage-dependent growth in the fibroblast cell cycle. J Cell Biol 115:1419–1452.

Guadagno TM, Ohtsubo M, Roberts JM, Assoian RK. 1993. A link between cyclin A expression and adhesion-dependent cell cycle progression. Science 262:1572–1575.

Hasenstab D, Forough R, Clowes AW. 1997. Plasminogen activator inhibitor type 1 and tissue inhibitor of metalloproteinases-2 increase after arterial injury in rats. Circ Res 80:490–496.

Hawks K, Higgins PJ. 1998. Cell shape-dependent pathway of plas-

minogen activate inhibitor type-1 gene expression requires cytoskeletal reorganization. J Cell Physiol 176:293–302.
Higgins PJ, Ryan MP. 1992. Identification of the 52 kDa cytoskeletal protein of cytochalasin D-stimulated normal rat kidney (NRK/CD) cells as substrate-associated glycoprotein p52 [plasminogen-activator inhibitor type-1 (PAI-1)]: expression of p52(PAI-1) in NRK/CD cells is regulated at the level of mRNA abundance. Biochem J 284:433–439.
Higgins PJ, Ryan MP, Chaudhari P. 1989. Cytochalasin D-mediated hyperinduction of the substrate-associated 52-kilodalton protein p52 in rat kidney fibroblasts. J Cell Physiol 139:407–417.
Higgins PJ, Ryan MP, Zeheb R, Gelehrter TD, Chaudhari P. 1990. p52 induction by cytochalasin D in rat kidney fibroblasts: homologies between p52 and plasminogen activator inhibitor type-1. J Cell Physiol 143:321–329.
Higgins PJ, Chaudhari P, Ryan MP. 1991. Cell shape regulation and matrix protein p52 content in phenotypic variants of ras-transformed rat kidney fibroblasts. Biochem J 273:651–658.
Higgins PJ, Ryan MP, Providence KM. 1994. Induced expression of p52(PAI-1) in normal rat kidney cells by the microfilament-disrupting agent cytochalasin D. J Cell Physiol 159:187–195.
Higgins PJ, Ryan MP, Jelley DM. 1997. $p52^{PAI-1}$ gene expression in butyrate-induced flat revertants of v-ras-transformed rat kidney cells: mechanism of induction and involvement in the morphological response. Biochem J 321:431–437.
Howell AL, Hunt JA, James TW, Mazar A, Henkin J, Zacharski LR. 1994. Urokinase inhibits HL-60 cell proliferation in vitro. Blood Coagul Fibrinolysis 5:445–453.
Jensen PJ, Lavker RM. 1996. Modulation of the plasminogen activator cascade during enhanced epidermal proliferation *in vivo*. Cell Growth Differ 7:1793–1804.
Kanse SM, Kost C, Wilhelm OG, Andreasen PA, Preissner KT. 1996. The urokinase receptor is a major vitronectin-binding protein on endothelial cells. Exp Cell Res 224:344–353.
Kariko K, Kuo A, Boyd D, Okada S, Cines DB, Barnathan ES. 1993. Overexpression of urokinase receptor increases matrix invasion without altering cell migration in a human osteosarcoma cell line. Cancer Res 53:3109–3117.
Kawada M, Umezawa K. 1995. Suppression of in vitro invasion of human fibrosarcoma cells by a leupeptin analogue inhibits the urokinase-plasmin system. Biochem Biophys Res Commun 209:25–30.
Kjoller L, Kanse SM, Kirkegaard T, Rodenburg KW, Ronne E, Goodman SL, Preissner KT, Ossowski L, Andreasen PA. 1997. Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. Exp Cell Res 232:420–429.
Krakoff IH, Brown NC, Reichard P. 1968. Inhibition of ribonucleotide diphosphate reductase by hydroxurea. Cancer Res 28:1559–1565.
Kutz SM, Nickey S, White LA, Higgins PJ. 1997. Induced PAI-1 mRNA expression and targeted protein accumulation are early G_1 events in serum-stimulated rat kidney cells. J Cell Physiol 170:8–18.
Laiho M, Keski-Oja J. 1989. Growth factors in the regulation of pericellular proteolysis: a review. Cancer Res 49:2533–2553.
Ledger PW, Uchida N, Tanzer ML. 1980. Imunocytochemical localization of procollagen and fibronectin in human fibroblasts: effects of the monovalent ionophore, monensin. J Cell Biol 87:663–671.
Liu G, Shuman MA, Cohen RL. 1995. Co-expression of urokinase, urokinase receptor and PAI-1 is necessary for optimum invasion of cultured lung cancer cells. Int J Cancer 60:501–506.
Loskutoff DJ, Curriden SA, Hu G, Deng G. 1999. Regulation of cell adhesion by PAI-1. APMIS 107:54–61.
McGuire PG, Alexander SM. 1993. Inhibition of urokinase synthesis and cell surface binding alters the motile behavior of embryonic endocardial-derived mesenchymal cells in vitro. Development 118: 931–939.
Meissauer A, Kramer MD, Hofmann M, Erkell LJ, Jocob E, Schirrmacher V, Brunner G. 1991. Urokinase-type and tissue-type plasminogen activators are essential for in vitro invasion of human melanoma cells. Exp Cell Res 192:453–459.
Mimuro J, Loskutoff DJ. 1989a. Binding of type 1 plasminogen activator inhibitor to the extracellular matrix of cultured bovine endothelial cells. J Biol Chem 264:5058–5063.
Mimuro J, Loskutoff DJ. 1989b. Purification of a protein from bovine plasma that binds to type 1 plasminogen activator inhibitor and prevents its interaction with extracellular matrix: evidence that the protein is vitronectin. J Biol Chem 264:936–939.
Mu XC, Staiano-Coico L, Higgins PJ. 1998. Increased transcription and modified growth state-dependent expression of the plasminogen activator inhibitor type-1 gene characterize the senescent phenotype in human diploid fibroblasts. J Cell Physiol 174:90–98.
Okada SS, Tomaszewski JE, Barnathan ES. 1995. Migrating vascular smooth muscle cells polarize cell surface urokinase receptor after injury *in vitro*. Exp Cell Res 217:180–187.
Okedon LE, Sato Y, Rifkin DB. 1992. Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial migration independent of its proteolytic activity. J Cell Physiol 150:258–263.
Pappot H, Gardsvoll H, Romer J, Pedersen AN, Grondahl-Hansen J, Pyke C, Brunner N. 1995. Plasminogen activator inhibitor type 1 in cancer: therapeutic and prognostic implications. J Biol Chem 376: 259–267.
Pawar S, Kartha S, Toback FG. 1995. Differential gene expression in migrating renal epithelial cells after wounding. J Cell Physiol 165: 556–565.
Pepper MS, Vassalli JD, Montesano R, Orci L. 1987. Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. J Cell Biol 105:2535–2541.
Pepper MS, Sappino AP, Montesano R, Orci L, Vassalli JD. 1992. Plasminogen activator inhibitor-1 is induced in migrating endothelial cells. J Cell Physiol 153:129–139.
Pepper MS, Sappino AP, Stocklin R, Montesano R, Orci L, Vassalli JD. 1993. Upregulation of urokinase receptor expression on migrating endothelial cells. J Cell Biol 122:673–684.
Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG, Parks WC. 1997. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. J Cell Biol 137:1445–1457.
Planus E, Barlovatz-Mermon G, Rodgers RA, Bonavaud S, Ingber DE, Wang N. 1997. Binding of urokinase to plasminogen activator inhibitor type-1 mediates cell adhesion and spreading. J Cell Sci 110:1091–1098.
Pollanen J, Saksela O, Salonen EM, Andreasen P, Neilsen L, Dano K, Vaheri A. 1987. Distinct localization of urokinase-type plasminogen activator and its type-1 inhibitor under cultured human fibroblasts and sarcoma cells. J Cell Biol 104:1085–1096.
Pollanen J, Ross W, Vaheri A. 1991. Directed plasminogen activation at the surface of normal and transformed cells. Adv Cancer Res 57:273–328.
Reidy M, Irwin C, Lindner V. 1995. Migration of arterial wall cells. Circ Res 78:405–414.
Romer J, Lund LR, Eriksen J, Ralfkiaer E, Zeheb R, Gelehrter TD, Dano K, Kristensen P. 1991. Differential expression of urokinase-type plasminogen activator and its type-1 inhibitor during healing of mouse skin wounds. J Invest Dermatol 97:803–811.
Romer J, Bugge TH, Pyke C, Lund LR, Flick MJ, Degen JL, Dano K. 1996. Impaired wound healing in mice with a disrupted plasminogen gene. Nat Med 2:287–292.
Ryan MP, Higgins PJ. 1988. Cytoarchitecture of Kirsten sarcoma virus-transformed rat kidney fibroblasts: butyrate-induced reorganization within the actin microfilament network. J Cell Physiol 137:25–34.
Ryan MP, Higgins PJ. 1993. Growth state-regulated expression of p52(PAI-1) in normal rat kidney cells. J Cell Physiol 155:376–384.
Ryan MP, Kutz SM, Higgins PJ. 1996. Complex regulation of plasminogen activator inhibitor type-1 (PAI-1) gene expression by serum and substrate adhesion. Biochem J 314:1041–1046.
Sato Y, Rifkin DB. 1988. Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. J Cell Biol 107:1199–1205.
Schafer BM, Maier K, Eickhoff U, Todd RF, Kramer MD. 1994. Plasminogen activation in healing human wounds. Am J Pathol 144: 1269–1280.
Schneiderman J, Sawdey M, Craig H, Thinnes T, Bordui G, Loskutoff DJ. 1993. Type 1 plasminogen activator inhibitor gene expression following partial hepatectomy. Am J Pathol 143:753–762.
Seiffert D, Mimuro J, Schleef RR, Loskutoff DJ. 1990. Interactions between type 1 plasminogen activator inhibitor, extracellular matrix and vitronectin. Cell Differ Dev 32:287–292.
Smith TJ, Ahmed A, Hogg MG, Higgins PJ. 1992. Interferon-gamma is an inducer of plasminogen activator inhibitor type 1 in human orbital fibroblasts. Am J Physiol 263:C24–C29.
Stahl A, Mueller BM. 1994. Binding of urokinase to its receptor promotes migration and invasion of human melanoma cells in vitro. Cancer Res 54:3066–3071.
Staiano-Coico I, Carano K, Allan VM, Steiner MG, Pagan-Charry I, Bailey BB, Babaar P, Rigas B, Higgins PJ. 1996. PAI-1 gene expression is growth state-regulated in cultured human epidermal keratinocytes during progression to confluence and postwounding. Exp Cell Res 227:123–134.
Stefansson S, Lawrence DA. 1996. The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha v\beta 5$ binding to vitronectin. Nature 383:441–443.

Thornton AJ, Bruzdzinski CJ, Raper SE, Gelehrter TD. 1994. Plasminogen activator inhibitor-1 is an immediate early response gene in regenerating rat liver. Cancer Res 54:1337–1343.

Uchida N, Smilowitz H, Ledger PW, Tanzer ML. 1980. Kinetic studies of the intracellular transport of procollagen and fibronectin in human fibroblasts: effects of the monovalent ionophore, monensin. J Biol Chem 255:8638–8644.

Underwood PA, Steele JG, Dalton BA. 1993. Effects of polystyrene surface chemistry on the biological activity of solid phase fibronectin and vitronectin, analyzed with monoclonal antibodies. J Cell Sci 104:793–803.

Uno S, Nakamura M, Seki T, Ariga T. 1997. Induction of tissue-type plasminogen activator (tPA) and type-1 plasminogen activator inhibitor (PAI-1) as early growth responses in rat hepatocytes in primary culture. Biochem Biophys Res Commun 239:123–128.

Vassalli JD, Sappino AP, Belin D. 1991. The plasminogen activator/plasmin system. J Clin Invest 88:1067–1072.

Wallin A, Zhang G, Jones TW, Jaken S, Stevens JL. 1992. Mechanisms of the nephrogenic repair process: studies on proliferation and vimentin expression after ^{35}S-1,2-dichlorovinyl-L-cysteine nephrotoxicity in vivo and in cultured proximal tubule epithelial cells. Lab Invest 66:474–484.

Waltz DA, Natkin LR, Fujita RM, Wei Y, Chapman HA. 1997. Plasmin and plasminogen activator inhibitor type-1 promote cellular motility by regulating the interaction between the urokinase receptor and vitronectin. J Clin Invest 100:58–67.

Wang PJ, Chabes A, Casagrande R, Tian XC, Thelander L, Huffaker TC. 1997. Rnr4p, a novel ribonucleotide reductase small-subunit protein. Mol Cell Biol 17:6114–6121.

Wei Y, Waltz DA, Rao N, Drummond RJ, Rosenberg S, Chapman HA. 1994. Identification of the urokinase receptor as an adhesion receptor for vitronectin. J Biol Chem 51:32380–32388.

Wei Y, Lukashev M, Simon DI, Bodary SC, Rosenberg S, Doyle MV, Chapman HA. 1996. Regulation of integrin function by the urokinase receptor. Science 273:1551–1555.

White LA, Bruzdzinski C, Gelehrter TD, Higgins PJ. 1999. Transcriptional regulation of plasminogen activator inhibitor type-1 gene expression in renal epithelial cells by a proximal promoter E box element. J Cell Physiol (in press).

Wilcox SA, Reho T, Higgins PJ, Tominna-Sebald E, McKeown-Longo PJ. 1996. Localization of urokinase to focal adhesions by human fibrosarcoma cells synthesizing recombinant vitronectin. Biochem Cell Biol 74:899–910.

Woods A, Couchman JR. 1988. Focal adhesions and cell-matrix interactions. Coll Relat Res 8:155–182.

Yamada KM, Clark RAF. 1995. Provisional matrix. In: Clark RAF, editor. The molecular and cellular biology of wound repair. New York: Plenum Press. p 51–93.

Zahm JM, Kaplan H, Herard AL, Doriot F, Pierrot D, Somelette P, Puchelle E. 1997. Cell migration and proliferation during the in vitro wound repair of the respiratory epithelium. Cell Motil Cytoskeleton 37:33–43.